

## PCT COOPERATION TREATY

PCT

## NOTIFICATION RELATING TO PRIORITY CLAIM

(PCT Rules 26bis.1 and 26bis.2 and  
Administrative Instructions, Sections 402 and 409)

From the INTERNATIONAL BUREAU

To:

PLOUGMANN, VINGTOFT & PARTNERS A/S  
Sankt Annae Plads 11  
P.O. Box 3007  
DK-1021 Copenhagen K  
DANEMARK

Date of mailing (day/month/year) 21 July 1999 (21.07.99)	
Applicant's or agent's file reference 20935 PC 1	<b>IMPORTANT NOTIFICATION</b>
International application No. PCT/DK99/00258	International filing date (day/month/year) 07 May 1999 (07.05.99)
Applicant GX BIOSYSTEMS A/S et al	

The applicant is hereby **notified** of the following in respect of the priority claim(s) made in the international application.

1. ☒ **Correction of priority claim.** In accordance with the applicant's notice received on: 16 July 1999 (16.07.99), the following priority claim has been corrected to read as follows:

US 12 May 1998 (12.05.98) 60/085,067

- ☐ even though the indication of the number of the earlier application is missing.  
☐ even though the following indication in the priority claim is not the same as the corresponding indication appearing in the priority document:

2. ☐ **Addition of priority claim.** In accordance with the applicant's notice received on: , the following priority claim has been added:

- ☐ even though the indication of the number of the earlier application is missing.  
☐ even though the following indication in the priority claim is not the same as the corresponding indication appearing in the priority document:

3. ☐ As a **result of the correction and/or addition** of (a) priority claim(s) under items 1 and/or 2, the (earliest) priority date is:

4. ☐ **Priority claim considered not to have been made.**

- ☐ The applicant failed to respond to the Invitation under Rule 26bis.2(a) (Form PCT/IB/316) within the prescribed time limit.  
☐ The applicant's notice was received after the expiration of the prescribed time limit under Rule 26bis.1(a).  
☐ The applicant's notice failed to correct the priority claim so as to comply with the requirements of Rule 4.10.

The applicant may, before the technical preparations for international publication have been completed and subject to the payment of a fee, request the International Bureau to publish, together with the international application, information concerning the priority claim. See Rule 26bis.2(c) and the PCT Applicant's Guide, Volume I, Annex B2(IB).

5. ☒ In case where **multiple priorities** have been claimed, the above item(s) relate to the following priority claim(s):

US 12 May 1998 (12.05.98) 60/085,067

6. A copy of this notification has been sent to the receiving Office and

- ☒ to the International Searching Authority (where the international search report has not yet been issued).  
☒ the designated Offices (which have already been notified of the receipt of the record copy).

<p>The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland</p> <p>Facsimile No. (41-22) 740.14.35</p>	<p>Authorized officer</p> <p>Aino Metcalfe</p> <p>Telephone No. (41-22) 338.83.38</p>
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WO 99/58652  
PCT/DK99/00258

## PATENT COOPERATION TREATY

PCT/PCT Rec'd 07 NOV 2000

PCT

NOTICE INFORMING THE APPLICANT OF THE  
COMMUNICATION OF THE INTERNATIONAL  
APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

From the INTERNATIONAL BUREAU

To:

PLOUGMANN, VINGTOFT & PARTNERS A/S  
Sankt Annae Plads 11  
P.O. Box 3007  
DK-1021 Copenhagen K  
DANEMARK  
PLOUGMANN  
VINGTOFT  
& PARTNERS

26 NOV. 1999

FIV/LPD

Date of mailing (day/month/year) 18 November 1999 (18.11.99)		
Applicant's or agent's file reference 20935 PC 1		
IMPORTANT NOTICE		
International application No. PCT/DK99/00258	International filing date (day/month/year) 07 May 1999 (07.05.99)	Priority date (day/month/year) 07 May 1998 (07.05.98)
Applicant GX BIOSYSTEMS A/S et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:

AU,CN,EP,IL,JP,KP,KR,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GD,GE,GH,GM,HR,  
HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,  
SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW

The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on 18 November 1999 (18.11.99) under No. WO 99/58652

## REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

## REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 740.14.35	Authorized officer J. Zahra Telephone No. (41-22) 338.83.38
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ENT COOPERATION TREA

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C.20231  
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

<b>Date of mailing</b> (day/month/year) 22 November 1999 (22.11.99)	
<b>International application No.</b> PCT/DK99/00258	<b>Applicant's or agent's file reference</b> 20935 PC 1
<b>International filing date</b> (day/month/year) 07 May 1999 (07.05.99)	<b>Priority date</b> (day/month/year) 07 May 1998 (07.05.98)
<b>Applicant</b> GERDES, Kenn et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:  
12 October 1999 (12.10.99)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was  
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<p>The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland</p> <p>Facsimile No.: (41-22) 740.14.35</p>	<p>Authorized officer</p> <p>Jean-Marie McAdams</p> <p>Telephone No.: (41-22) 338.83.38</p>
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The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/

PCT Rec'd 07 NOV 2000

PCT

VOY/BA

## CHAPTER II

## DEMAND

under Article 31 of the Patent Cooperation Treaty:

The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA		Date of receipt of DEMAND	
<b>Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION</b>		Applicant's or agent's file reference 20935 PC 1	
International application No. PCT/DK99/00258	International filing date (day/month/year) 7 May 1999	(Earliest) Priority date (day/month/year)	
Title of invention Cytotoxin-based biological containment			
<b>Box No. II APPLICANT(S)</b>			
Name and address: (Familyname followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)  GX BioSystems A/S Forskerbyen Symbion Fruebjergvej 3 DK 2100 Copenhagen Ø		Telephone No.:	
		Facsimile No.:	
		Teleprinter No.:	
State (that is, country) of nationality: Denmark		State (that is, country) of residence: Denmark	
Name and address: (Familyname followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)  GERDES, Kenn Majsmarken 17 DK-5260 Odense S			
State (that is, country) of nationality: Denmark		State (that is, country) of residence: Denmark	
Name and address: (Familyname followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)  GOTFREDSSEN, Marie Ørstedsgade 44, 2. th. DK-5000 Odense C			
State (that is, country) of nationality: Denmark		State (that is, country) of residence: Denmark	
<input checked="" type="checkbox"/> Further applicants are indicated on a continuation sheet.			



Sheet No. 2

International application No.  
PCT/DK99/00258

Continuation of Box No. II APPLICANT(S)

*If none of the following sub-boxes is used, this sheet should not be included in the demand.*

Name and address: (Familyname followed by givenname; for a legal entity, full official designation. The address must include postal code and name of country.)

GRØNLUND, Hugo  
Uffesvej 26  
DK-5200 Odense VState (that is, country) of nationality:  
DenmarkState (that is, country) of residence:  
Denmark

Name and address: (Familyname followed by givenname; for a legal entity, full official designation. The address must include postal code and name of country.)

PEDERSEN, Kim  
Solfaldsvej 18-11  
DK-5000 Odense CState (that is, country) of nationality:  
DenmarkState (that is, country) of residence:  
Denmark

Name and address: (Familyname followed by givenname; for a legal entity, full official designation. The address must include postal code and name of country.)

KRISTOFFERSEN, Peter  
Bispeengen 15, 1. tv.  
DK-2000 FrederiksbergState (that is, country) of nationality:  
DenmarkState (that is, country) of residence:  
Denmark

Name and address: (Familyname followed by givenname; for a legal entity, full official designation. The address must include postal code and name of country.)

State (that is, country) of nationality:

State (that is, country) of residence:



Further applicants are indicated on another continuation sheet.

Sheet No. 3

International application No.  
PCT/DK99/00258

## Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representativeand ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.Name and address: (Family name followed by given name; for a legal entity, full official designation.  
The address must include postal code and name of country.)Plougmann, Vingtoft & Partners A/S  
Sankt Annæ Plads 11  
p.o. Box 3007  
DK-1021 Copenhagen K

Telephone No.:

+45 33 63 93 00

Facsimile No.:

+45 33 63 96 00

Teleprinter No.:

☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

## Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

## Statement concerning amendments:\*

1. The applicant wishes the international preliminary examination to start on the basis of:

☐ the international application as originally filedthe description ☒ as originally filed☐ as amended under Article 34the claims ☒ as originally filed☐ as amended under Article 19 (together with any accompanying statement)☐ as amended under Article 34the drawings ☒ as originally filed☐ as amended under Article 342. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.3. ☐ The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). (This check-box may be marked only where the time limit under Article 19 has not yet expired.)

\* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

☒ which is the language in which the international application was filed.☐ which is the language of a translation furnished for the purposes of international search.☐ which is the language of publication of the international application.☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

## Box No. V ELECTION OF STATES

The applicant hereby elects all eligible States (that is, all States which have been designated and which are bound by Chapter II of the PCT)

excluding the following States which the applicant wishes not to elect:

Sheet No. 4

International application No.

PCT/DK99/00258

## Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:

- |  |   |   |        |
|--|---|---|--------|
| 1. translation of international application                              | : |   | sheets |
| 2. amendments under Article 34   | : |   | sheets |
| 3. copy (or, where required, translation) of amendments under Article 19 | : |   | sheets |
| 4. copy (or, where required, translation) of statement under Article 19  | : |   | sheets |
| 5. letter  | : | 1 | sheets |
| 6. other (specify)   | : |   | sheets |

For International Preliminary Examining Authority use only

received	not received
----------	--------------

- |                          |                          |
|--------------------------|--------------------------|
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |

The demand is also accompanied by the item(s) marked below:


- |  |   |
|--|---|
| 1. <input checked="" type="checkbox"/> fee calculation sheet                             | 4. <input type="checkbox"/> statement explaining lack of signature                                  |
| 2. <input type="checkbox"/> separate signed power of attorney                            | 5. <input type="checkbox"/> nucleotide and or amino acid sequence listing in computer readable form |
| 3. <input type="checkbox"/> copy of general power of attorney; reference number, if any: | 6. <input type="checkbox"/> other (specify):  |

## Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

Copenhagen, 12 October 1999

Plougmann, Vingtoft &amp; Partners A/S

  
 Henry Sogaard

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

- |  |   |
|--|---|
| 3. <input type="checkbox"/> The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply.                        | <input type="checkbox"/> The applicant has been informed accordingly. |
| 4. <input type="checkbox"/> The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.                               |   |
| 5. <input type="checkbox"/> Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82. |   |

For International Bureau use only

Demand received from IPEA on:

**(PCT Rule 13bis)**

Form PCT/MO/134 (July 1967)

**INDICATIONS RELATING TO DEPOSITED MICROORGANISMS**  
(PCT Rule 12bis)

5 Additional sheet

In addition to the microorganism indicated on page 33 of the description, the following microorganisms have been deposited with

10 DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH  
Mascheroder Weg 1b, D-38124 Braunschweig, Germany

on the dates and under the accession numbers as stated below:

15

Accession number	Date of deposit	Description Page No.	Description Line No.
20 DSM 12158	30 April 1998	33	32
DSM 12159	30 April 1998	35	10
DSM 12160	30 April 1998	34	31
DSM 12161	30 April 1998	33	21

25

For all of the above-identified deposited microorganisms, the following additional indications apply:

30

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed

35 to be withdrawn.

PCT

REQUEST - 7 MAJ 1999

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference  
(if desired) (12 characters maximum) 20935 PC 1

## Box No. I TITLE OF INVENTION

CYTOTOXIN-BASED BIOLOGICAL CONTAINMENT

## Box No. II APPLICANT

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

GX BioSystems A/S  
Forskerbyen Symbion  
Fruebjergvej 3  
DK-2100 Copenhagen Ø

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State (that is, country) of nationality:

DK

State (that is, country) of residence:

DK

This person is applicant for the purposes of:

☐ all designated States☒ all designated States except the United States of America☐ the United States of America only☐ the States indicated in the Supplemental Box

## Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

GERDES, Kenn  
Majsmarken 17  
DK-5260 Odense S

This person is:

☐ applicant only☒ applicant and inventor☐ inventor only (If this check-box is marked, do not fill in below.)

State (that is, country) of nationality:

DK

State (that is, country) of residence:

DK

This person is applicant for the purposes of:

☐ all designated States☐ all designated States except the United States of America☒ the United States of America only☐ the States indicated in the Supplemental Box☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

## Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:

☒ agent☐ common representative

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Plougmann, Vingtoft & Partners A/S  
Sankt Annae Plads 11  
P.O. Box 3007  
DK-1021 Copenhagen K

Telephone No.

+ 45 33 63 93 00

Facsimile No.

+ 45 33 63 96 00

Teleprinter No.

☐ Address for correspondence: Mark this check box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Sheet No. ...2....

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
<i>If none of the following sub-boxes is used, this sheet should not be included in the request.</i>	
<p>Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</p> <p>GOTFREDSEN, Marie Ørstedsgade 44, 2. th DK-5000 Odense C</p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input checked="" type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only (If this check-box is marked, do not fill in below.)</p>
State (that is, country) of nationality: DK	State (that is, country) of residence: DK
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p>Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</p> <p>GRØNLUND, Hugo Uffesvej 26 DK-5200 Odense V</p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input checked="" type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only (If this check-box is marked, do not fill in below.)</p>
State (that is, country) of nationality: DK	State (that is, country) of residence: DK
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p>Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</p> <p>PEDERSEN, Kim Solfaldsvej 18-11 DK-5000 Odense C</p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input checked="" type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only (If this check-box is marked, do not fill in below.)</p>
State (that is, country) of nationality: DK	State (that is, country) of residence: DK
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p>Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</p> <p>Peter Kristoffersen Bispeengen 15, 1. tv DK-2000 Frederiksberg</p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input checked="" type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only (If this check-box is marked, do not fill in below.)</p>
State (that is, country) of nationality: DK	State (that is, country) of residence: DK
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p><input type="checkbox"/> Further applicants and/or (further) inventors are indicated on another continuation sheet.</p>	

Sheet No. 3

**Box No.V DESIGNATION OF STATES**

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

**Regional Patent**

- ☒ **AP** ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ **EA** Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP** European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ **OA** OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line) .....

National Patent (if other kind of protection or treatment desired, specify on dotted line):

- |   |   |
|---|---|
| <input checked="" type="checkbox"/> <b>AL</b> Albania .....                               | <input checked="" type="checkbox"/> <b>LS</b> Lesotho .....                                   |
| <input checked="" type="checkbox"/> <b>AM</b> Armenia .....                               | <input checked="" type="checkbox"/> <b>LT</b> Lithuania .....                                 |
| <input checked="" type="checkbox"/> <b>AT</b> Austria ..and utility model...              | <input checked="" type="checkbox"/> <b>LU</b> Luxembourg .....                                |
| <input checked="" type="checkbox"/> <b>AU</b> Australia .....                             | <input checked="" type="checkbox"/> <b>LV</b> Latvia .....                                    |
| <input checked="" type="checkbox"/> <b>AZ</b> Azerbaijan .....                            | <input checked="" type="checkbox"/> <b>MD</b> Republic of Moldova .....                       |
| <input checked="" type="checkbox"/> <b>BA</b> Bosnia and Herzegovina .....                | <input checked="" type="checkbox"/> <b>MG</b> Madagascar .....                                |
| <input checked="" type="checkbox"/> <b>BB</b> Barbados .....                              | <input checked="" type="checkbox"/> <b>MK</b> The former Yugoslav Republic of Macedonia ..... |
| <input checked="" type="checkbox"/> <b>BG</b> Bulgaria .....                              | <input checked="" type="checkbox"/> <b>MN</b> Mongolia .....                                  |
| <input checked="" type="checkbox"/> <b>BR</b> Brazil .....                                | <input checked="" type="checkbox"/> <b>MW</b> Malawi .....                                    |
| <input checked="" type="checkbox"/> <b>BY</b> Belarus .....                               | <input checked="" type="checkbox"/> <b>MX</b> Mexico .....                                    |
| <input checked="" type="checkbox"/> <b>CA</b> Canada .....                                | <input checked="" type="checkbox"/> <b>NO</b> Norway .....                                    |
| <input checked="" type="checkbox"/> <b>CH and LI</b> Switzerland and Liechtenstein .....  | <input checked="" type="checkbox"/> <b>NZ</b> New Zealand .....                               |
| <input checked="" type="checkbox"/> <b>CN</b> China .....                                 | <input checked="" type="checkbox"/> <b>PL</b> Poland .....                                    |
| <input checked="" type="checkbox"/> <b>CU</b> Cuba .....                                  | <input checked="" type="checkbox"/> <b>PT</b> Portugal .....                                  |
| <input checked="" type="checkbox"/> <b>CZ</b> Czech Republic ..and utility model...       | <input checked="" type="checkbox"/> <b>RO</b> Romania .....                                   |
| <input checked="" type="checkbox"/> <b>DE</b> Germany ..and utility model...              | <input checked="" type="checkbox"/> <b>RU</b> Russian Federation .....                        |
| <input checked="" type="checkbox"/> <b>DK</b> Denmark ..and utility model...              | <input checked="" type="checkbox"/> <b>SD</b> Sudan .....                                     |
| <input checked="" type="checkbox"/> <b>EE</b> Estonia ..and utility model...              | <input checked="" type="checkbox"/> <b>SE</b> Sweden .....                                    |
| <input checked="" type="checkbox"/> <b>ES</b> Spain .....                                 | <input checked="" type="checkbox"/> <b>SG</b> Singapore .....                                 |
| <input checked="" type="checkbox"/> <b>FI</b> Finland ..and utility model...              | <input checked="" type="checkbox"/> <b>SI</b> Slovenia .....                                  |
| <input checked="" type="checkbox"/> <b>GB</b> United Kingdom .....                        | <input checked="" type="checkbox"/> <b>SK</b> Slovakia ..and utility model...                 |
| <input checked="" type="checkbox"/> <b>GD</b> Grenada .....                               | <input checked="" type="checkbox"/> <b>SL</b> Sierra Leone .....                              |
| <input checked="" type="checkbox"/> <b>GE</b> Georgia .....                               | <input checked="" type="checkbox"/> <b>TJ</b> Tajikistan .....                                |
| <input checked="" type="checkbox"/> <b>GH</b> Ghana .....                                 | <input checked="" type="checkbox"/> <b>TM</b> Turkmenistan .....                              |
| <input checked="" type="checkbox"/> <b>GM</b> Gambia .....                                | <input checked="" type="checkbox"/> <b>TR</b> Turkey .....                                    |
| <input checked="" type="checkbox"/> <b>HR</b> Croatia .....                               | <input checked="" type="checkbox"/> <b>TT</b> Trinidad and Tobago .....                       |
| <input checked="" type="checkbox"/> <b>HU</b> Hungary .....                               | <input checked="" type="checkbox"/> <b>UA</b> Ukraine .....                                   |
| <input checked="" type="checkbox"/> <b>ID</b> Indonesia .....                             | <input checked="" type="checkbox"/> <b>UG</b> Uganda .....                                    |
| <input checked="" type="checkbox"/> <b>IL</b> Israel .....                                | <input checked="" type="checkbox"/> <b>US</b> United States of America .....                  |
| <input checked="" type="checkbox"/> <b>IN</b> India .....                                 | <input checked="" type="checkbox"/> <b>UZ</b> Uzbekistan .....                                |
| <input checked="" type="checkbox"/> <b>IS</b> Iceland .....                               | <input checked="" type="checkbox"/> <b>VN</b> Viet Nam .....                                  |
| <input checked="" type="checkbox"/> <b>JP</b> Japan .....                                 | <input checked="" type="checkbox"/> <b>YU</b> Yugoslavia .....                                |
| <input checked="" type="checkbox"/> <b>KE</b> Kenya .....                                 | <input checked="" type="checkbox"/> <b>ZW</b> Zimbabwe .....                                  |
| <input checked="" type="checkbox"/> <b>KG</b> Kyrgyzstan .....                            |   |
| <input checked="" type="checkbox"/> <b>KP</b> Democratic People's Republic of Korea ..... |   |
| <input checked="" type="checkbox"/> <b>KR</b> Republic of Korea .....                     |   |
| <input checked="" type="checkbox"/> <b>KZ</b> Kazakhstan .....                            |   |
| <input checked="" type="checkbox"/> <b>LC</b> Saint Lucia .....                           |   |
| <input checked="" type="checkbox"/> <b>LK</b> Sri Lanka .....                             |   |
| <input checked="" type="checkbox"/> <b>LR</b> Liberia .....                               |   |

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- ☒ **AE** United Arab Emirates .....
- ☒ **ZA** South Africa .....
- ☐ .....

**Precautionary Designation Statement:** In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)



Sheet No. 4

Box No. VI PRIORITY CLAIM				
Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country	regional application: <sup>a</sup> regional Office	International application: receiving Office
item (1) 7 May 1998 07.05.98	PA 1998 00627	DK		
item (2) 12 May 1998 12.05.98	60/085,067	US		
item (3)				

☐ Further priority claims are indicated in the Supplemental Box.

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present International application is the receiving Office) identified above as item(s): (1)

<sup>a</sup> Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(h)(i)). See Supplemental Box.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY			
Choice of International Searching Authority (ISA) (if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):	Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority):		
ISA /EP	Date (day/month/year)	Number	Country (or regional Office)
	16 December 1998	RS 100951	DK EP

Box No. VIII CHECK LIST: LANGUAGE OF FILING	
<p>This international application contains the following number of sheets:</p> <p>request : 4</p> <p>description (excluding sequence listing part) : 70</p> <p>claims : 12</p> <p>abstract : 1</p> <p>drawings : 22</p> <p>sequence listing part of description : 20</p> <p>Total number of sheets : 129</p>	<p>This international application is accompanied by the item(s) marked below:</p> <p>1. <input checked="" type="checkbox"/> fee calculation sheet</p> <p>2. <input type="checkbox"/> separate signed power of attorney</p> <p>3. <input type="checkbox"/> copy of general power of attorney; reference number, if any:</p> <p>4. <input type="checkbox"/> statement explaining lack of signature</p> <p>5. <input type="checkbox"/> priority document(s) identified in Box No. VI as item(s):</p> <p>6. <input type="checkbox"/> translation of international application into (language):</p> <p>7. <input type="checkbox"/> separate indications concerning deposited microorganism or other biological material</p> <p>8. <input checked="" type="checkbox"/> nucleotide and/or amino acid sequence listing in computer readable form</p> <p>9. <input type="checkbox"/> other (specify):</p>
<p>Figure of the drawings which should accompany the abstract: 4</p>	<p>Language of filing of the international application: English</p>

Box No. IX SIGNATURE OF APPLICANT OR AGENT	
<p>Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).</p> <p>Copenhagen, 7 May 1999</p> <p>Plougmann, Vingtoft &amp; Partners A/S</p> <p>Henry Sogaard</p>	

For receiving Office use only	
1. Date of actual receipt of the purported international application:	<p>2. Drawings:</p> <p><input type="checkbox"/> received:</p> <p><input type="checkbox"/> not received:</p>
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority (if two or more are competent): ISA /	
6. <input type="checkbox"/> Transmittal of search copy delayed until search fee is paid.	

For International Bureau use only	
Date of receipt of the record copy by the International Bureau:	

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>20935 PC 1</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/DK 99/ 00258</b>	International filing date (day/month/year) <b>07/05/1999</b>	(Earliest) Priority Date (day/month/year) <b>07/05/1998</b>
Applicant <b>GX BIOSYSTEMS A/S et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

### 1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☒ contained in the international application in written form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☒ **Unity of invention is lacking** (see Box II).

### 4. With regard to the title,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

### 5. With regard to the abstract,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

### 6. The figure of the drawings to be published with the abstract is Figure No.

☒ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

4

☐ None of the figures.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 99/ 00258

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

SEE ADDITIONAL SHEET

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-79, 87, 88 all complete; 80-86, 89 all partially

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

1. Claims: (1-79, 87, 88 all complete); (80-86, 89 all partially)

A method for conditionally controlling cell survival, a method of confining an extrachromosomal replicon to a cell population, a method of post-segregationally stabilizing a plasmid in a cell population, a microbial cell, a composition comprising a cell, a method of limiting the survival of a cell population comprising the RelE gene family.

2. Claims: (80-86, 89 all partially)

A method for limiting the survival of a cell population comprising CcdB.

3. Claims: (80-86, 89 all partially)

same as in invention 2 but comprising PemK.

4. Claims: (80-86, 89 all partially)

same as in invention 2 but comprising ParE.

5. Claims: (80-86, 89 all partially)

same as in invention 2 but comprising Doc.

## INTERNATIONAL SEARCH REPORT

National Application No

PCT/DK 99/00258

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BERNARD P ET AL: "POSITIVE-SELECTION VECTORS USING THE F PLASMID CCDB KILLER GENE" GENE, vol. 148, no. 1, 1 January 1994 (1994-01-01), pages 71-74, XP000578378 the whole document	80-86,89
A	--- SALMON M A ET AL.: "The antidote and autoregulatory functions of the F plasmid CcdA protein: a genetic and biochemical survey" MOLECULAR AND GENERAL GENETICS, vol. 244, no. 5, 1 September 1994 (1994-09-01), pages 530-538, XP002088012 the whole document --- -/--	80-86,89



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## ° Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

16 August 1999

Date of mailing of the international search report

22. 11. 99

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Oderwald, H

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BECH F W ET AL.: "Sequence of the relB transcription unit from Escherichia coli and identification of the relB gene" EMBO JOURNAL, vol. 4, no. 4, 1985, pages 1059-1066, XP002087936 cited in the application abstract; figure 3</p> <p>---</p>	
A	<p>JENSEN R B AND GERDES K: "Programmed cell death in bacteria: proteic plasmid stabilization systems" MOLECULAR MICROBIOLOGY, vol. 17, no. 2, 1995, pages 205-210, XP002087937 cited in the application the whole document</p> <p>---</p>	
A	<p>SAUL D ET AL.: "Nucleotide sequence and replication characteristics of RepFIB, a basic replicon of IncF plasmids" JOURNAL OF BACTERIOLOGY, vol. 171, no. 5, May 1989 (1989-05), pages 2697-2707, XP002087938 abstract; figure 1</p> <p>---</p>	
A	<p>FLEISCHMANN R D ET AL: "WHOLE-GENOME RANDOM SEQUENCING AND ASSEMBLY OF HAEMOPHILUS INFLUENZAE RD" SCIENCE, vol. 269, no. 5223, 28 July 1995 (1995-07-28), pages 496-498, 507 - 512, XP000517090 the whole document</p> <p>---</p>	
A	<p>MANNING P A: "V. cholerae DNA for RVC repeated sequence (AC No. X64097)" EMBL SEQUENCE DATABASE, 7 August 1993 (1993-08-07), XP002087993 Heidelberg, Germany see ORF (9 kDa), complement (ntd. 1908-2156) the whole document &amp; FRANZON V L ET AL.: INFECTION AND IMMUNITY, vol. 61, no. 7, 1993, pages 3032-3037, XP002087943</p> <p>---</p>	
A	<p>TOMB J -F ET AL: "THE COMPLETE GENOME SEQUENCE OF THE GASTRIC PATHOGEN HELICOBACTER PYLORI" NATURE, vol. 388, 7 August 1997 (1997-08-07), pages 539-547, XP002070312 the whole document</p> <p>---</p> <p style="text-align: center;">-/--</p>	

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 99/00258

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KANEKO T ET AL.: "Sequence analysis of the genome of the unicellular cyanobacterium Synechocystis sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions" DNA RESEARCH, vol. 3, 1996, pages 109-136, XP002087939 the whole document ---	
A	BAUM J A: "Tn5401, a new class II transposable element from Bacillus thuringiensis" JOURNAL OF BACTERIOLOGY, vol. 176, no. 10, May 1994 (1994-05), pages 2835-2845, XP002087942 the whole document ---	
A	BULT C J ET AL.: "Complete genome sequence of the methanogenic archaeon, Methanococcus jannaschii" SCIENCE, vol. 273, 23 August 1996 (1996-08-23), pages 1058-1073, XP002087940 cited in the application the whole document ---	
P,X	GOTFREDSEN M AND GERDES K: "The Escherichia coli relBE genes belong to a new toxin-antitoxin gene family" MOLECULAR MICROBIOLOGY, vol. 29, no. 4, August 1998 (1998-08), pages 1065-1076, XP002087944  the whole document ---	1-3, 8-18,26, 29-31, 33-35, 40,41, 47,48, 50, 80-85,89
P,X	GRONLUND H AND GERDES K: "Toxin-antitoxin systems homologous with relBE of Escherichia coli plasmid P307 are ubiquitous in prokaryotes" JOURNAL OF MOLECULAR BIOLOGY, vol. 285, no. 4, 29 January 1999 (1999-01-29), pages 1401-1415, XP002112332 the whole document ---	1-20,26, 29-41, 47-55, 60-85,89
T	COLE S T ET AL.: "Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence" NATURE, vol. 393, 11 June 1998 (1998-06-11), pages 537-544, XP002087941 the whole document -----	

16  
RECD 21 FEB 2000

WIPO PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 20935 PC 1	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/DK99/00258	International filing date (day/month/year) 07/05/1999	Priority date (day/month/year) 07/05/1998
International Patent Classification (IPC) or national classification and IPC C12N15/00		
Applicant GX BIOSYSTEMS A/S et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 4 sheets, including this cover sheet.

- ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand  12/10/1999	Date of completion of this report  15.02.00
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Bretherick, J  Telephone No. +49 89 2399 8415 



# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/DK99/00258

## I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

### Description, pages:

1-92 as originally filed

### Claims, No.:

1-89 as originally filed

### Drawings, sheets:

1/22-22/22 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

## III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 80-86 and 89 all partially.

because:

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/DK99/00258

- ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☒ no international search report has been established for the said claims Nos. 80-86 and 89 all partially.

## V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

### 1. Statement

Novelty (N)	Yes:	Claims	1-79,87,88 (80-86 and 89 partially)
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-79,87,88 (80-86 and 89 partially)
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-79,87,88 (80-86 and 89 partially)
	No:	Claims	

### 2. Citations and explanations

see separate sheet

## VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

---

International application No. PCT/DK99/00258

1. Regarding Part VIII, Art. 5 and 6 PCT:
  - a. The claimed subject-matter refers to a functionally equivalent polypeptide of RelE. This is not clear under Art. 6 PCT, since the definition given of functional equivalent as the relE gene family is not itself defined in terms of technical features, either in the claims or in the art available.
  - b. The application does not demonstrate the existence of functional equivalents of relE, pointing out only sequence homology at the DNA level in numerous Genera and Phyla. There is no demonstration that the respective DNA also encodes functional equivalents to relE in these other groups and species of organisms. In this respect, the subject-matter of claims 2-7, 17, 30-32, 34-39, 41, 47-79, 87-89 are considered to be speculative and thus deficient in terms of Art. 5 PCT, since these mention the uses thereof in such groups.
2. Regarding Part V, Art. 33 PCT:
  - a. This Report has been written on the assumption that the priority right assigned to the corresponding document US 60/085,067, filed 12/05/98 holds for the entire subject-matter of this application.
  - b. There is no mention in the cited art or in the material given in the application **per se** of any use of the E. coli Rel.E gene product in recombinant methods of biological containment of a microbial cell population. In as far as the subject-matter can be interpreted, novelty and an inventive step might be acknowledged.



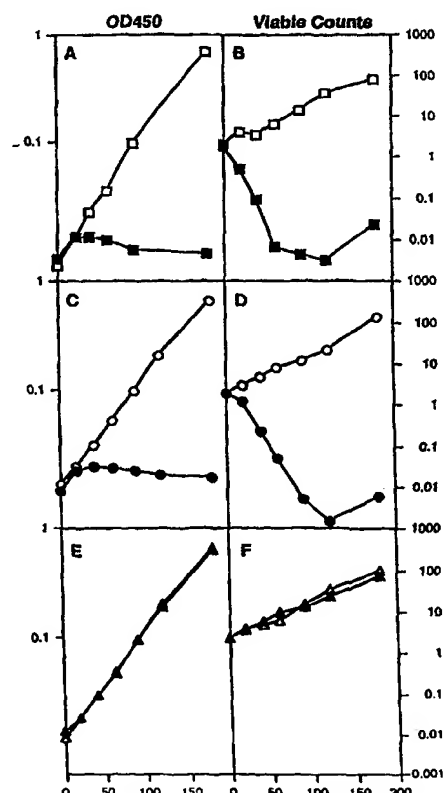
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/00</b>		<b>A2</b>	(11) International Publication Number: <b>WO 99/58652</b>
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(54) Title: CYTOTOXIN-BASED BIOLOGICAL CONTAINMENT

## (57) Abstract

Method of conditionally controlling the survivability of a recombinant cell population and of containing such cells to an environment or containing replicons to a host cell is based on the use of proteic killer systems including the *E. coli relBE* locus and similar systems found in Gram-negative and Gram-positive bacteria and *Archae*. Such system are generally based on a cytotoxin polypeptide and an antitoxin or antidote polypeptide that in contrast to the cytotoxin is degradable by proteases. The recombinant cells are useful as vaccines, pollutant degrading organisms or as biological pest control organisms e.g. expressing *B. thuringiensis* crystalline proteins.



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## CYTOTOXIN-BASED BIOLOGICAL CONTAINMENT

## FIELD OF THE INVENTION

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The present invention relates to the field of biologically containing genetically modified microorganisms in a particular environment and vectors to a particular host cell. Specifically, there is provided recombinant vectors and cells containing a proteic killer system based on the *E. coli* RelE polypeptide and functional equivalents of this cytotoxin and method of containing replicons and cells, respectively to particular host cells or particular environments, respectively.

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## TECHNICAL BACKGROUND AND PRIOR ART

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The increasing application of recombinant DNA technology to engineer novel microorganism which are industrially useful have caused concerns in the general public over the potential risks involved. These concerns are primarily related to the potential harm to humans and to undesirable and/or uncontrollable ecological consequences upon de-  
liberate or unintentional release of such genetically engineered microorganisms (GEMs) into the environment. These concerns have led to the establishment of official guidelines for the safe handling of GEMs in laboratories and production facilities where such organisms are applied. Up till now, such guidelines have primarily been directed to measures of physically containing GEMs in laboratories and production facilities with  
the aim of reducing the likelihood that workers in such facilities were contaminated, or that the GEMs were to escape from their primary physical environment, such as a fermentation vessel.

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It is presently being recognized that the level of safety in the handling of GEMs can be increased by combining physical containment measures with biological containment measures to reduce the possibility of the survival of the genetically engineered organisms if they were to escape from their primary environment.

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Lately, however, concerns have become increasingly focused on potential risks related to deliberate release of GEMs to the outer environment and to the use of GEMs as live vaccines. In this connection there is a strongly felt need to have biological containment systems which subsequent to the environmental release of the GEMs or their administration as vaccines to a human or an animal body, effectively kill the released organisms in a controlled manner or which limit the function of the released GEMs to an extent where such GEMs are placed at a significant competitive disadvantage whereby they will eventually be ousted by the natural microflora of the environment to which they are released.

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The first systems of biological containment were based on the use of "safe" cloning vectors and debilitated host bacteria. As examples, it has been suggested to select vectors which lack transfer functions or which naturally have a very narrow host range. Examples of debilitated host bacteria are *E. coli* mutants having an obligate requirement for exogenous nutrients not present or present in low concentrations outside the primary environment of the GEMs.

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Other suggested biological containment systems have been based on mechanisms whereby the vector is restricted to the GEMs e.g. by using a plasmid vector with a nonsense mutation in a gene, the expression of which is indispensable for plasmid replication or a suppressor mutation in the chromosome, said mutation blocking translational read-through of the message of the gene. A further approach is to maintain the rDNA stably in the host by integrating it into the chromosomes of the GEMs.

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Recently, an alternative biological containment strategy has been developed in which a recombinant vector is endowed with a gene encoding a cell killing function which gene is under the control of a promoter only being expressed under certain environmental conditions, such as conditions prevailing in an environment outside the primary environment of the GEMs, or when the vector is unintentionally transferred to a secondary host, or the expression of which is stochastically induced. By using incorporation in a GEM of such a cell killing function and selecting appropriate regulatory sequences, vectors can be constructed which are contained in the primary host cell and/or in a primary physical environment. A cell killing function as defined herein may also be referred to as an active biological containment factor.

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If a stochastically induced mechanism of expression regulation is selected for such a biological containment system, a population of GEMs containing the system will, upon release to the outer environment or if used as a live vaccine, be subjected to a random cell killing which will lead to an increase of the doubling time of the host cell population or eventually to the disappearance of the organisms.

The above-mentioned genes encoding cell killing functions are also frequently referred to as "suicide" genes, and biological containment systems based upon the use of such genes, the expression of which are regulated as defined above, are commonly described as conditional lethal systems or "suicide" systems. Up till now, several such cell killing functions have been found in bacterial chromosomes and in prokaryotic plasmids. Examples of chromosomal genes having cell killing functions are the *gef* (Poulsen et al., 1990) and *relF* (Bech et al., 1985) genes from *E. coli* K-12. Examples of plasmid encoded suicide genes are *hok* and *flmA* (Gerdes et al., 1986) genes isolated from plasmids R1 and F, respectively, the *snrB* gene also isolated from plasmid F (Akimoto et al., 1986) and the *pnd* gene isolated from plasmids R16 and R483 (Sakikawa et al., 1989 and Ono et al., 1987). Common features of these genes are that they are transcribed constitutively, regulated at a post-transcriptional level, and that they all encode small toxic proteins of about 50 amino acids and that their translation is controlled by antisense RNA. The application of the *hok* gene in a biological containment system has been disclosed in WO 87/05932.

An alternative biological containment system is disclosed in WO 95/10614 which is based on the use of genes, the expression of which in a cell where the gene is inserted, results in the formation of mature forms of exoenzymes which are hydrolytically active in the cytoplasm of the cell and which can not be transported over the cell membrane. When such enzymes are expressed, the normal function of the cell becomes limited to an extent whereby the competitiveness, and hence the survival, of a population of such cells is reduced.

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The stable maintenance of low copy-number plasmids in bacteria is secured by a number of plasmid-borne gene systems, one of which is based on killing of plasmid-free cells (also termed post-segregational killing). This regulated killing is based on a toxin-antidote principle, i.e. a two-component system comprising a stable toxin and an un-



stable antidote for the toxin. One such system, which is referred to as a proteic killer gene system is based on protein toxins and protein antidotes (reviewed by Jensen and Gerdes, 1995). The natural function of such systems is to provide stable maintenance of plasmids and it has not been suggested previously to utilize the systems as the basis for confining GEMs to a particular environment.

The *E. coli relB* operon encodes three genes, *relB*, *relE* and *relF* (Bech et al., 1985). It has now been found that *relE* encodes a cytotoxin whose overproduction is lethal to host cells and that the *relB* gene encodes an antitoxin that prevents the lethal action of RelE. When present on a plasmid, the *relBE* operon was able to stabilize the inheritance of a mini-R1 test plasmid. It was also found that *relBE* homologous gene systems are found in a wide variety of Gram-negative and Gram-positive bacteria and in *Archae*.

These results show that the *relBE* genes constitute a new ubiquitously occurring family of gene systems that belongs to the proteic plasmid stabilization systems.

These findings has opened up for an alternative, highly effective and versatile biological containment system as it is described in the following. Importantly, it has been discovered that such a system involves the significant advantage that the frequency of spontaneously occurring mutants of microorganisms that have become resistant to the lethal effect of these cytotoxins is very low. This implies that this biological containment system is very safe.

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## SUMMARY OF THE INVENTION

Accordingly, the invention pertains in a first aspect to a method of conditionally controlling the survivability of a recombinant microbial cell population, the method comprising (i) providing in the cells of said population a gene coding for a cytotoxic first kind of polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally equivalent polypeptide (said genes collectively being designated herein as the *relE* gene family), said gene is expressible in the cells of the population and, operably linked to the gene,

a regulatable regulatory DNA sequence and (ii) cultivating the cell population under conditions where the *relE* gene or the gene coding for a functionally equivalent polypeptide is expressed, the expression leading to an at least partial killing of the cell population.

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In a further aspect there is provided a method of confining an extrachromosomal replicon to a microbial cell population, the method comprising the steps of

(i) isolating a microbial cell naturally containing a gene belonging to the *relE* gene family coding for a first kind of polypeptide that, when it is expressed in the cell, acts as a toxin for the cell or, if the cell does not naturally contain a gene belonging to the *relE* gene family, introducing such a gene into the cell,

(ii) introducing into the cell the extrachromosomal replicon to be confined, said replicon containing a gene coding for a second kind of polypeptide that, by binding to the first kind of polypeptide, acts as an antitoxin for said first kind of polypeptide,

(iii) cultivating the cell under conditions where the genes coding for the first and the second kind of polypeptides are expressed, whereby a daughter cell that does not receive a copy of the extrachromosomal replicon is killed by the first kind of polypeptide being expressed in the absence of expression of the second kind of polypeptide.

In a still further aspect, the invention relates to a method of post-segregationally stabilizing a plasmid in a microbial host cell population, the method comprising the steps of

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(i) recombinationally inserting into the plasmid (a) a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a functional equivalent thereof, said first kind of polypeptide having a toxin effect on the host cell and (b) a gene coding for a second kind of polypeptide that (1) is capable of acting as an antitoxin for first kind of polypeptide and (2) is capable of being degraded in the host cell at a higher rate than that at which the first kind of polypeptide is degraded,

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(ii) cultivating the cell population under conditions where the genes coding for the first kind and second kind of polypeptides are expressed,

whereby a daughter cell that does not receive at least one copy of the plasmid is killed  
5 as a result of the faster degradation of the second kind of polypeptide.

In yet other aspects, the invention provides a recombinant microbial cell comprising a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, a gene coding for a functionally equivalent polypeptide  
10 hereof or a variant or derivative of any such polypeptide, said first kind of polypeptide having a toxic effect on the cell, subject to the limitation that when the cell is *E. coli*, the gene coding for the first kind of polypeptide is not derived from *E. coli*, and a composition comprising such cells.

15 The invention also pertains to several methods of containing cells or replicons including

(1) a method of limiting the survival of a cell population in a first or a second environment, which method comprises

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(i) transforming the cells of said population with a gene coding for a cytotoxic polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4  
25 ParE polypeptide, the gene coding for the prophage P1 Doc polypeptide and a gene coding for a functionally equivalent polypeptide for anyone of said polypeptides, said gene is expressible in the cells of the population, and operably linked to the gene, a regulatory DNA sequence being regulatable by an environmental factor and which regulates the expression of said gene, and

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(ii) cultivating the cell population under environmental conditions where the gene coding for the cytotoxic polypeptide is expressed, the expression leading to an at least partial killing of the cell population,

(2) a method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is naturally transferable to a second kind of cell, which method comprises providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides to an extent whereby the function of the cell is being limited, said first kind of cells having or being modified to have a chromosomal replicon comprising a regulatory nucleotide sequence the gene product of which inhibits the expression of said gene or the cell function-limiting effect of the polypeptide and thereby protects said first kind of cells, said regulatory gene being lacking in said second kind of cell, whereby, if a cell of the second kind receives said extrachromosomal recombinant replicon said gene is expressed and has a function-limiting effect on said second kind of cell, and

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(3) a method of stochastically limiting in an environment the survival of a cell population, the method comprising transforming the cells thereof with a recombinant replicon containing a regulatably expressible gene which, when expressed in a cell, encodes a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides, the expression of said gene leading to formation of the polypeptide to an extent whereby the function of the cells is being limited, the expression of said gene is stochastically induced as a result of re-combinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cells, inhibits expression of the gene coding for the polypeptide, said negatively functioning regulatory nucleotide sequence being contained in the recombinant replicon or in an other recombinant replicon present in cells of the population containing the replicon.

## DETAILED DISCLOSURE OF THE INVENTION

One objective of the present invention is to provide a novel approach to conditionally controlling the survivability of a recombinant microbial cell population. This approach  
5 is based on the use of what is generally referred to as proteic killer systems which have been reviewed i.a. by Jensen et al., 1995. These systems consist of two components, a cytotoxin polypeptide (also referred to herein as a first kind of polypeptide) and a corresponding antitoxin or antidote polypeptide (also referred to herein as a second kind of polypeptide) that by binding to the cytotoxic polypeptide inhibits the toxic  
10 effect hereof. A general characteristic of such proteic killer systems is that the antitoxin component in contrast to the toxin component is susceptible to protease degradation, resulting in a decay of the antitoxin polypeptide.

As used herein, the expression "microbial cell" includes any prokaryotic and eukaryotic  
15 cells as well as cells of *Archae* species. Thus this expression includes cells of bacterial species, fungal species, animal species including invertebrates, vertebrates, mammals, humans and insects, and plant cells.

Thus, in one aspect of the invention there is provided such a method of conditionally  
20 controlling the survivability of a recombinant microbial cell population that comprises as the first step, providing in the cells a gene coding for a cytotoxic first kind of polypeptide, which gene is selected from the gene coding for the *E. coli* K-12 RelE polypeptide and a gene coding for a structurally and functionally equivalent polypeptide and, operably linked to the gene, a regulatable regulatory DNA sequence.

25 Genes which are structurally and functionally equivalent to *relE* are herein collectively referred to as the *relE* gene family or as *relE* homologues. This group of genes including the *E. coli* plasmid P307 derived *relE* homologue encompasses genes the gene products of which have cytotoxic effects and which, relative to *E. coli* K-12 RelE,  
30 have at least 20% such as at least 30% e.g. at least 40% identical and conserved amino acids. The sequences listed in the below Table 1.5 are putative RelE homologues.

Whereas, in accordance with the invention, presently preferred recombinant microbial cells are prokaryotic cells such as Gram-negative and Gram-positive bacterial cells, it has been found that the survivability of other microbial cells such as *Archae*, yeast cells, fungal cells, animal cells including human cells and plant cells and replicons of such organisms can be conditionally controlled using the methods of the present invention.

In the present context, the expression "conditionally controlling" refers to a construction of the microbial cell which permits that the gene coding for the cytotoxic polypeptide can be expressed under certain pre-determined environmental conditions whereas under other such conditions, the gene is not expressed. Hence, the survivability of the microbial cells can be made dependent on certain pre-selected conditions.

In accordance with the invention, the survivability of microbial cells is controlled by the expression in the cells of a cytotoxic polypeptide selected from *E. coli* K-12 RelE polypeptide and a functionally equivalent polypeptide. As used herein, the term "cytotoxic" refers not only to a loss of the ability of microbial cells containing the toxin-encoding gene to remain viable as determined by the capability to propagate in media which, under identical environmental conditions, support unrestricted growth of similar cells not containing the toxin-encoding gene, but also to cells having, as a result of the expression of the polypeptide-encoding gene, a limited cell function, the latter expression denoting that the growth of a cell as manifested i.a. by the synthesis of new cell material and the rate of replication of the cell is decreased.

During the experimentation leading to the invention it was surprisingly found that a range of cytotoxic polypeptides according to the invention have the effect that they inhibit translation of genes. This general effect of RelE polypeptides and functionally equivalent polypeptides appears to represent a hitherto undiscovered mechanism for controlling survivability of cells and thus for containment of such cells or replicons in accordance with the methods of the present invention.

Whereas the recognizable manifestation of such limited cell function may ultimately be cell death, it may also be a reduced cell growth appearing as a reduced rate of replication resulting in a reduced increase of cell numbers within a certain period of time as a

result of an increase of the lag phase and/or of the cell doubling time. Other manifestations may be a relatively increased requirement for one or more nutrient components or a relatively higher susceptibility to detrimental environmental factors such as sub-optimal temperatures or cell damages caused by toxic substances.

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In the present context, the expression "a functionally equivalent polypeptide" refers to a polypeptide that has substantially the same effect on the survivability of microbial cells as the RelE polypeptide of *E. coli* K-12. As it is shown herein, a variety of Gram-positive and Gram-negative bacteria and *Archae* organisms comprise DNA sequences showing homology with the RelE polypeptide. To the extent gene products of structural homologues of the *relE* gene product show an effect on microbial cell survivability as it is defined above, they are encompassed by the present invention. It will also be appreciated that the term "functional equivalent" includes variants or derivatives of any of the above first kind of polypeptides, the sequences of which have been modified by substitution, deletion or addition of one or more amino acids and the gene product of which has retained at least part of the cytotoxic function of the gene product of the non-modified sequence.

In the above method, a regulatable regulatory DNA sequence is operably linked to the gene coding for the cytotoxic polypeptide. In accordance with the invention, such a regulatory sequence can be one with which the gene coding for the cytotoxic polypeptide is naturally associated or it can be a sequence with which the gene is not naturally associated. In the present context, the term "regulatory DNA sequence" is intended to indicate a DNA sequence which directly or indirectly regulates the expression of the gene coding for the cytotoxic polypeptide at the level of transcription or at the level of translation or at the level of protein function. The regulatory DNA sequence may thus be one, the function of which results in a suppression or inhibition of the activity of the regulatable promoter.

Such regulatory DNA sequences are referred to herein as "negatively functioning regulatory DNA sequences". One interesting example of such a regulatory DNA sequence is a sequence coding for a repressor substance which represses the expression of the gene coding for cytotoxically active polypeptide and which substance may, when a cell containing it is released to a human or an animal body or to the outer envi-

ronment where the substance is no longer being expressed, undergo a decay whereby the repression of expression of the cytotoxin-encoding gene is gradually reduced and eventually, when the decay of the repressor is completed, the repression is removed.

- 5 Another example of such a regulatory DNA sequence is a sequence encoding a polypeptide that acts as an antidote or antitoxin for the cytotoxic polypeptide. Such a sequence include the *relB* gene derived from the *relBE* operon of *E. coli* K-12 which is capable of binding to the RelE polypeptide and thereby inhibiting its effect. As also shown herein, sequences encoding such antitoxins can be found in Gram-negative and
- 10 Gram-positive bacteria and in *Archae*. Such homologues of the *relB* sequence are encompassed by the present invention.

In preferred embodiments of the invention, the regulatory DNA sequence may be present in the cell in one or more recombinant replicons and it may be contained in the

15 same replicon as that containing the cytotoxin-encoding gene or in a different recombinant replicon.

One way whereby the expression of the cell function-limiting cytotoxic polypeptide can be regulated is by providing in the cell a gene coding for the polypeptide, which

20 gene is regulated at the level of transcription. The regulation at the level of transcription may be carried out in various ways including a regulation by means of a promoter, regulated by one or more factors. These factors may either be ones which by their presence ensure expression of the gene coding for polypeptide or may, alternatively, be factors which suppress the expression of the gene so that their absence causes the

25 polypeptide to be expressed.

Factors regulating the activity of the promoter as defined above may be selected from a variety of factors. Thus, the expression of the gene encoding the polypeptide may be determined by the environmental conditions, by the physiological state of the cells,

30 or by a cyclical or stochastic event. In the present context, the term "cyclical event" is understood to mean a cyclically recurrent event causing changes in certain factors known to be potentially useful in affecting the expression of genes such as temperature conditions, changes in light intensity or hormonal changes. The term "physiologi-



cal state of the cells" denotes factors such as cell density or the growth phase of cells.

In accordance with the invention, advantageous promoter regulating factors are readily regulatable factors including the presence or absence of a certain chemical substance in the environment or the physical conditions in the environment such as the prevailing temperature or other physical factors (e.g. the intensity of the light in the environment). Thus, it is possible to envisage containment systems as presently claimed, in which the gene coding for the cytotoxic polypeptide is expressed when a certain chemical substance present in a first environment such as the fermentation medium in which the cell is propagated, is not present in a second environment to which the cell is released, or when a factor required for the growth or survival of the cell is no longer present, or the factor is one which, when it is depleted or exhausted from an environment of the cell, has the desired effect, viz. that the gene is expressed.

The promoter regulating the transcription of the gene coding for the cytotoxic polypeptide can also become activated in a second environment of the cell by a chemical substance which is not present in a first environment of the cell, but which is present in the second environment in sufficient quantities to activate the promoter. Similarly, the promoter may be a promoter which is activated by a shift in temperature, such as a shift from a higher temperature in a first environment as e.g. a fermentation vessel, to a lower temperature prevailing in an outside second environment, or the intensity of light, in that the promoter may be one which is activated in the presence of light of sufficient intensity, but is inactive in the darkness prevailing in a first environment such as a fermentation vessel.

Where microbial cells as defined herein are cells that are to be released to the outer environment in a controlled manner, e.g. to a restricted area of land or to the intestinal tract of a human or an animal, the regulatable promoter may be one which is regulated chemically, i.e. by the presence or absence of a certain chemical substance in the environment of the cells as it has been explained above.

However, the regulatable promoter is advantageously a promoter which is activated cyclically, e.g. by changes of the temperature, or by a stochastic event. The term

- "stochastic event" as used herein is intended to denote an event which occurs at random at a certain frequency per cell per generation or frequency per unit time which, in accordance with the invention, may result in a limitation of the function of the cells in which the activation of expression of the cytotoxic polypeptide occurs, optionally to an extent which leads to the death of the cells. The stochastic event may be occasioned by periodic inversions of the region carrying the promoter or by recombinational excision of a recombinationally excisable negatively functioning regulatory DNA sequence as defined above.
- 10 It should be noted that in order to ensure a general applicability of the present invention, the promoter used to initiate transcription of the gene coding for the toxic polypeptide is preferably a promoter which is capable of causing expression of said gene in a wide range of cells.
- 15 In case of regulatable transcription of the polypeptide, the regulatory DNA sequence may e.g. be a promoter isolated from bacterial operons involved in the biosynthesis of amino acids or from bacterial genes, the transcription of which is activated late in the stationary growth phase or from bacterial genes involved in the synthesis of cell surface structures such as fimbriae. Examples of suitable promoters include the *E. coli trp* promoter which becomes activated in the absence of tryptophan, the bacteriophage  $\lambda$   $P_R$  and  $P_L$  promoters controlled by temperature sensitive regulatory DNA sequences, the *Bacillus subtilis* sporulation gene promoters which are activated during sporulation, and the *E. coli* and *Salmonella* fimbriae gene promoters which are activated stochastically.
- 25 In case of chemically regulatable promoters, the chemical substance, the presence or absence of which determines the activation of the promoter, is suitably selected from carbon or nitrogen sources, metabolites, amino acids, nucleosides, purine or pyrimidine bases or metal ions. When the chemical substance is one which, when present, suppresses promoter activity, it is preferably a substance which rarely occurs in the natural environment in such concentrations that the promoter would not be activated when the cell is released to the natural environment. One example of such a promoter is the *trp* promoter which is repressed in the presence of tryptophan in the environment of the cell, but which is derepressed in the absence of sufficient amounts of
- 30

tryptophan in the environment. A containment system according to the invention using the *trp* promoter or another promoter being regulated in the same manner, might therefore comprise an amount of tryptophan in a first environment, such as a fermentation vessel, which is sufficient to repress the promoter in such an environment, the  
5 promoter, however, being derepressed when the cell is released from the first environment to a second environment, e.g. the outer environment which usually contains very low amounts of tryptophan or no tryptophan at all.

It is also possible to select a promoter that is regulated by the absence or presence of  
10 one or more compounds in exudates of plants colonized with a recombinant organism according to invention.

In this context, another useful promoter is an arabinose inducible promoter including that contained in the plasmid pBAD (Guzman et al., 1995). Without arabinose added  
15 to the growth medium, the pBAD promoter is completely turned off. However, in the presence of arabinose, strong transcription is induced. This particular promoter is repressible by the addition of glucose to the growth medium. Thus, by the addition of glucose, transcription from pBAD can be rapidly and efficiently turned off. The glucose repression effect is epistatic to the inducer effect by arabinose. Hence, if cells with a  
20 pBAD-carrying plasmid are grown in a medium containing both arabinose and glucose, the promoter is not induced. However, if cell growth depletes the medium for glucose, then the promoter will be induced. Therefore, such a plasmid is suitable for the conditional turning on and off the expression of gene, in particular toxin-encoding genes as described herein.

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Accordingly, in one embodiment of the invention the method is used to contain microbial cells wherein the promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound whereby, when the first kind of compound is depleted from the medium, the promoter is induced by the second kind  
30 of compound.

Another example of a regulatable promoter, the activation of which is determined by a chemical substance is the *lac* promoter which is inducible by e.g. isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG).

As mentioned above, the regulatable promoter may be a promoter, the activity of which is determined by the temperature prevailing in the environment of a cell containing the gene coding for the cell function-limiting cytotoxin and a regulatable promoter regulating the expression of the gene. In such a case, the regulation of the promoter is advantageously obtained by the presence in the cell of a gene coding for a temperature sensitive repressor for the promoter. As one typical example, the  $\lambda$  promoters including those mentioned above may be regulated by a temperature sensitive  $\lambda$  cl repressor.

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Promoters which are activated stochastically by periodic inversions of the promoter region (in the present context, such promoters are also termed as "invertible promoters" and "inversional switch promoters") and which are useful for the purposes of the present invention include as examples the *hin*, *cin* and *gin* promoters. One particularly useful invertible promoter is the *fimA* promoter which is one *E. coli* fimbriae promoter. The activation (inversional switch) of this promoter is regulated by the gene products of the two genes which for the present purposes is termed the "on" and the "off" genes, the on gene product inducing a switch from off (inactive) to on (active), and the off gene product inducing a switch from on to off. In a wild-type *E. coli* cell where the *fimA* gene and its associated promoter is present in one copy on the chromosome, the inversional switch occurs with a switching frequency of about one cell/1000 cells/generation. It is, however, possible to regulate the frequency of the inversional switch as required by regulating the dosage of expression of the *on* and *off* genes. This is e.g. effected by means of suitable promoters transcribing into the on and off genes. The frequency of transcription initiation by these promoters will then determine the relative dosage levels of the *on* and *off* gene products being formed.

In accordance with the invention, one particular method of stochastically regulating the expression of the gene coding for the toxic polypeptide is the induction of the gene expression as a result of recombinational excision of an excisable negatively functioning regulatory DNA sequence which, while present in the cell, inhibits expression of the gene. In the present context, the term "recombinational excision" refers to the result of a naturally occurring phenomenon of genetic recombination (cross-over) whereby DNA sequences in replicons, in a controlled process, pair, break and rejoin to

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form recombinant replicons by the sequential action of enzymes acting on the DNA. The frequency of recombinational events in a cell depends i.a. on the degree of homology between paired complementary nucleotide sequences and on the length of the complementary sequences. Thus, it has been shown that about 50 base pairs of homology may be required to obtain recombination in a bacterial cell.

When a negatively regulatory DNA sequence is inserted between directly repeated nucleotide sequences of a sufficient length in a recombinationally proficient cell which, in accordance with the invention contains a gene coding for the toxic polypeptide, recombination between the repeats results in the recombinational excision of the negatively regulatory DNA sequence permitting the gene to be expressed.

Accordingly, the phenomenon of recombinational excision implies that a DNA subsequence, i.e. the negatively regulatory DNA sequence, is excised from a longer DNA sequence through a recombination event. In essence, the longer DNA sequence is cleaved on either side of the subsequence and the fresh ends are joined, leaving out the subsequence. Recombination occurs between sufficient homologous flanking nucleotide subsequences. Thus, with DNA of the general structure W-X-Y-X-Z, X being a repeated sequence and Y being a negatively regulatory DNA sequence, this could recombine to form W-X-Z, with the Y subsequence being excised.

As mentioned above, the frequency of the recombination can be determined by varying the lengths of the repeats and/or the distance between the repeats. Furthermore, the frequency may be varied by using repeat sequences of varying homologies. Thus, nucleotide sequence repeats being 100% homologous and having a size which does not impair recombination will result in a high recombination frequency and hence, in a high frequency of recombinational excision of the negatively regulatory sequence, whereas mismatches within complementary sequences will reduce the recombination frequency depending on the degree of mismatch. As an example, it has been found that 10% divergence between nucleotide sequence repeats may reduce the recombination frequency 40-fold.

Accordingly, the microbial cell comprising the gene coding for the cytotoxic polypeptide may, in accordance with the invention, be a cell containing a regulatory DNA se-

quence which is a recombinationally excisable negatively functioning regulatory DNA sequence being flanked by a first flanking nucleotide sequence and a second flanking nucleotide sequence substantially homologous with the first flanking sequence. As used herein, the term "substantially homologous with" is used to indicate that the degree of homology is sufficient to result in a desired frequency of recombination. In certain embodiments it may, in order to obtain a desirable maximum frequency of recombination, be advantageous to use direct repeats, i.e. sequences being 100% homologous, whereas in other embodiments where a moderate degree of cell function limitation is desirable, it is appropriate to use repeats which are more or less heterologous, but still allowing a desirable lower frequency of recombination to occur. Accordingly, in the present context, the term "sufficiently homologous" is used to indicate a degree of homology between two flanking nucleotide sequence repeats which results in a desired frequency of recombinational events in a cell containing the gene coding for the toxin polypeptide and a negatively regulatory DNA sequence.

As it also has been mentioned above, the frequency of recombination depends on the lengths of the flanking sequences. In useful embodiments of the invention, flanking sequences are used which have a length being in the range of 100-5,000 base pairs. In certain preferred embodiments, it is advantageous to use flanking sequences, the length of which is in the range of 200-3,000 base pairs. As the flanking sequences can be used any nucleotide repeats of sufficient lengths and homology as it has been defined above. As one useful example of flanking sequences may be mentioned the chloramphenicol resistance gene having a size of about 900 base pairs and which occurs in the plasmid pBR325. Another example of a useful nucleotide sequence which, when inserted as repeats, results in recombination, is a subsequence of the *rrnB* gene isolated from the plasmid pKK3535 (Brosius et al., 1981, Plasmid, 6:112-118) having a size e.g. in the range of 500 to about 3,000 base pairs, such as 598 base pairs.

In one interesting embodiment of the invention, the excisable negatively regulatory DNA sequence operably linked to the gene encoding the cytotoxic polypeptide is a gene encoding an antisense RNA which forms an RNA-RNA duplex with said the messenger RNA of the polypeptide-encoding gene and thereby, when it is expressed, inhibits translation of said gene coding for the polypeptide.

In another useful embodiment of the present invention, the recombinationally excisable negatively regulatory DNA sequence is a gene encoding a polypeptide repressor of transcription of the polypeptide-encoding gene. Such a repressor may, e.g. be a *lac* repressor including the repressor encoded by the *LacI* gene. It will be appreciated  
5 that the negatively regulatory DNA sequence can also be a gene coding for RelB anti-toxin or functionally equivalents hereof.

In a further useful embodiment of the invention, the excisable negatively regulatory DNA sequence is a transcription termination sequence, preventing the transcription of  
10 the cytotoxic polypeptide-encoding gene. In one specific embodiment of the invention, such a suitable terminator sequence is the *rpoCt'* transcription terminator isolated from the plasmid pHBA102rpoCt (Squires et al., 1981, Nucleic Acid Res., **9**:6827-6839).

Negatively regulatory DNA sequences which, in accordance with the invention, are  
15 suitable, can be isolated from DNA sequences derived from a virus, or a prokaryotic or eucaryotic cell. Thus, sources of the DNA sequence include bacterial chromosomes, bacterial plasmids, prokaryotic viruses, eucaryotic viruses, eucaryotic plasmids, or eucaryotic chromosomes.

20 In preferred embodiments of the invention, the excisable negatively regulatory DNA sequence and the first and second flanking sequences, both as defined above, is provided in the form of a "cassette" which term is used herein to describe a readily insertable DNA sequence comprising at least the above-mentioned sequences and optionally the gene coding for the cytotoxically active polypeptide, and optionally further  
25 nucleotide sequences including as examples a suitable marker such as a gene coding for antibiotic resistance. In the present context, the term "insertable" denotes that the cassette as defined herein is provided with suitable restriction sites at both ends allowing for insertion in a replicon having the same restriction sites. Accordingly, such preferred restriction sites include sites which occur frequently in replicons where inser-  
30 tion is desirable or alternatively, restriction sites which may be easily provided in such replicons.

It will be understood that, in accordance with the invention, a cassette as defined above and which does not comprise the gene coding for toxin polypeptide and opera-

bly linked to the negatively regulatory DNA sequence, may be inserted in a replicon which is different from the replicon containing said gene. Optionally, the cassette as defined above is inserted in a first replicon such as e.g. a transposon and subsequently inserted via the transposon into the chromosome to obtain a cell as defined herein.

5

As it has been explained above, the activation of certain invertible promoters such as the *fimA* promoter or functional homologues hereof is regulated by the gene products of an *on* gene and an *off* gene. It will be understood that this mechanism of promoter regulation provides the possibility of using the *off* gene or a functional homologue

10 hereof as a negatively regulatory DNA sequence which may be inserted in the microbial cell as defined herein, as a recombinationally excisable DNA sequence in the manner explained in details above. Accordingly, in one embodiment, the present invention provides a microbial cell wherein the toxin-encoding gene is stochastically expressed as a result of recombinational inversion of an invertible promoter sequence.

15

In plasmids, inherent mechanisms occur whereby multimer resolution of the plasmid during replication takes place. As exemplified by the broad host range plasmid RP4, this resolution system may comprise (1) a gene coding for a multimer resolving enzyme, a resolvase and (2) a site for the site-specific resolvase-mediated resolution. In

20 plasmid RP4 the gene coding for the resolvase is *parA* and the site for the resolution is designated *mrs*. If two *mrs* sites are placed in direct orientation, a DNA sequence inserted between those two sites may, if the *parA* gene is present in the same host cell, be deleted at a relatively high frequency whereby a site-specific recombination system is provided. In useful embodiments the *parA* gene may be located in trans.

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It has been found that such a site-specific recombination system provides a useful mechanism for stochastically regulating the expression of a gene such as the gene coding for the toxic polypeptide as defined herein, since the site-specific recombination may be used to obtain recombinational excision of a negatively regulatory DNA

30 sequence as defined above.

Accordingly, in one interesting embodiment, the present invention provides a microbial cell as defined herein in which the negatively regulatory DNA sequence is a sequence flanked by a first site for a site-specific resolution recombinase and a second site for



site-specific resolution, the second site being recognizable by the same or a functionally equivalent multimer resolving enzyme as is the first site, whereby the regulatory sequence is recombinationally excisable in the cell. In a specific embodiment, the gene coding for the multimer resolving enzyme is located *in trans* relative to the sites for  
5 site-specific resolution. In the present context, one useful example of a suitable gene is the *parA* gene isolated from plasmid RP4.

In accordance with the invention, the method of controlling the survivability of microbial cells can be based on providing in the cells a gene coding for a cytotoxic polypeptide that is structurally and functionally equivalent to the *E. coli* RelE polypeptide (the  
10 *relE* gene family). Such a gene can be derived from the chromosome or another replicon of a Gram-negative bacterium including *Enterobacteriaceae* spp. such as *E. coli*, *Hemophilus* spp. such as *H. influenzae*, *Vibrionaceae* spp. such as *V. cholerae*, *Pseudomonadaceae* spp., *Helicobacter* spp. such as *H. pylori* and *Synechosystis* spp, the  
15 latter organisms belonging to the group of cyanobacteria. The gene may also be derived from the chromosome and other replicons of Gram-positive bacteria including lactic acid bacteria such as *Streptococcus* spp including *Streptococcus pneumoniae*., *Bacillaceae* spp. such as *B. thuringiensis*, and *Mycobacterium* spp. and from species belonging to *Arhae* such as *Methanococcus jannaschii* and *A. fulgidus*. Such genes  
20 include those that are defined herein as belonging to the *relE* gene family. The RelE equivalent polypeptide from *M. jannaschii* was shown to be toxic for *E. coli* when expressed in this organism.

However, genes coding for cytotoxins of other proteic killer systems and which are  
25 therefore functional equivalents of the *E. coli* K-12 RelE polypeptide can also be used in accordance with the invention for conditionally controlling the survivability of microbial cells. Such genes include the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide and the gene coding for the prophage P1 Doc polypeptide, as de-  
30 scribed by Jensen et al., 1995.

It will be understood that in this context, the term "functional equivalent" includes variants or derivatives of any of the above first kind of polypeptides the sequences of which have been modified by substitution, deletion or addition of one or more amino

acids and the gene product of which has retained at least part of the function of the gene product of the non-modified sequence.

In accordance with the invention, the *relE* family gene or any gene coding for a toxin  
5 of a proteic killer system is provided in the microbial cells at a location where it can be expressed effectively. Thus, in useful embodiments the gene is present on the chromosome of the cells whereas in other embodiments it is preferably located on an extrachromosomal element such as a plasmid or a cosmid. In a specific embodiment, the microbial cells according to the invention do not contain a gene coding for a second  
10 type of polypeptide that is capable of counteracting the cell toxic effect of the RelE polypeptide or the functional equivalent hereof.

However, in other useful embodiments, the microbial cells comprise a gene coding for a second kind of polypeptide that is capable of binding to the *relE* polypeptide or the  
15 functional equivalent hereof, the binding resulting in that the toxic effect of the RelE polypeptide or the functional equivalent is at least partially counteracted. Such a counteracting second kind of polypeptide is, as it is mentioned above, also referred to herein as an antitoxin or an antidote for the cytotoxic polypeptide.

20 Although, in certain uses of the present method, it is preferred that the genes coding for both the toxic polypeptide and the antitoxin herefor is under the control of the same regulatory sequences, it may, in other uses, be advantageous that the gene coding for the second kind of polypeptide is operably linked to a different regulatable regulatory DNA sequence as defined above, permitting that the gene coding for the  
25 second kind of polypeptide is suppressed under conditions where the gene coding for the RelE polypeptide or the functional equivalent is expressed.

It will be appreciated that the genes coding for the toxin polypeptide and the antitoxin polypeptide, respectively can be present on the same replicon such as a plasmid or on  
30 the chromosome, or they can be present on different replicons in the microbial cells.

A useful second kind of polypeptide is the *RelB* polypeptide derived from *E. coli* K-12 which i.a. binds effectively to the *E. coli*-derived RelE polypeptide. However, the regulation of the toxic effect of the first kind of polypeptide can also be based on pro-

viding in the cells a gene coding for a second kind of antitoxically active polypeptide that is functionally equivalent to the *E. coli* RelB polypeptide. Such a gene can be derived from the chromosome or another replicon of a Gram-negative bacterium including *Enterobacteriaceae* spp. such as *E. coli*, *Hemophilus* spp. such as *H. influenzae*,  
5 *Vibrionaceae* spp. such as *V. cholerae*, *Pseudomonadaceae* spp., *Helicobacter* spp. such as *H. pylori*, and *Synechosystis* spp belonging to the group of cyanobacteria. Additionally, genes coding for structural and functional equivalents of the *E. coli* RelB polypeptide can be isolated from Gram-positive bacteria including lactic acid bacterial species such as *Streptococcus* spp., *Bacillaceae* spp. such as *B. thuringiensis*, and  
10 *Mycobacterium* spp. and from species belonging to *Arhae* such as *M. jannaschii* and *A. fulgidus*. Sequences for the *E. coli* RelB polypeptide and for equivalents isolated from the above organisms are listed in Table 1.6.

Genes coding for functional equivalents of the *E. coli* K-12 RelB polypeptide which in  
15 accordance with the invention can be used for containing microbial cells and replicons include the genes coding for the plasmid F CcdA polypeptide, the plasmid R1 PemI polypeptide, the plasmid RP4 ParD polypeptide and the prophage P1 Phd polypeptide.

It will be understood that in this context the term "functional equivalent" includes  
20 variants or derivatives of any of the above second kind of polypeptides, the sequences of which have been modified by substitution, deletion or addition of one or more amino acids and the gene product of which has retained at least part of the function of the gene product of the non-modified sequence.

25 It is a significant objective of the present invention to provide the means of conditionally controlling the survivability of microbial cells that expresses one or more genes coding for a gene product of interest. In accordance with the invention such an objective is pursued for any type of gene products including enzymes such as proteases, enzymes which are effective in degrading carbohydrates such as starch degrading en-  
30 zymes, lipid degrading enzymes and nucleases.

However, it is of particular interest to provide containment of microbial cells wherein the gene product of interest is selected from an immunologically active gene product,

a gene product that is effective in degradation of an environmental pollutant and a pesticidally active product.

Accordingly, in such specific embodiments the microbial cells are cells which further  
5 comprise a DNA sequence that is selected from a sequence coding for an immunologically active gene product, a sequence coding for a pesticidally active gene product and a sequence coding for a pollutant degrading gene product.

In the present context, the term "immunologically active gene product" is used to describe an epitope (antigenic determinant) from a pathogenic organism which, when it  
10 is administered to the body of a human or an animal, is capable of stimulating the formation of antibodies therein. A microbial cell as defined herein which contains one or more genes encoding such a gene product can be utilized in the preparation of live vaccines. In the immunization against several pathogens it is considered advantageous  
15 to administer live vaccines as compared to killed organisms or antigenic fragments of the pathogen, since the level of immunity conferred by a live vaccine is frequently higher than that conferred by vaccines comprising killed pathogenic organisms or fragments thereof. Most currently used vaccines comprising viable epitope-containing organisms are either based on recombinant non-pathogenic organisms encoding the  
20 epitope or they are based on attenuated pathogenic organisms. The cell advantageously contains a multiplicity of genes each of which codes for a specific immunologically active gene product.

However, up till now the use of live vaccines has been limited since it is difficult to  
25 obtain the right combination of attenuation, viability and adequate immune response. Furthermore, the deliberate release of genetically engineered microorganisms to the body and to the external environment which is a result of the use of viable recombinant organisms as vaccines, is currently not allowed in any country for reasons of public concern as to the possible long-term environmental impact, in particular the risk  
30 of permanent establishment of the GEMs in the environment.

The present invention provides advantageous means of circumventing these problems associated with the use of known GEM-based live vaccines by introducing into a viable epitope-containing cell the regulatably expressible gene coding for a cell toxic

polypeptide as defined above. In particularly interesting embodiments, the invention provides, as a useful basis for a viable vaccine, the microbial cells as defined above whose expression is stochastically induced.

- 5 In useful embodiments of the invention, the cell which contains the DNA sequence coding for an immunologically active gene product further comprises means for transporting the epitope, when expressed, to the outer surface of the cell, i.e. translocating it across the cell membrane. Preferably such a translocation is obtained by inserting the gene coding for the epitope into a nucleotide sequence coding for an outer cell
- 10 surface polypeptide structure such as fimbriae which contains the fimbrillin protein, pili, flagellae or certain other surface proteins including as an example the OM protein found in *Streptococcus* species. By providing the cell with such a hybrid nucleotide sequence being expressible in the cell, the gene product hereof will be a fusion or hybrid protein comprising the epitope and the relevant cell surface structure.

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- A cell in which a fusion protein is expressed which comprises the epitope fused to a surface structure protein by which the cell can adhere to the mucosal cells of a body to which the cell is administered is considered to be particularly useful in that the epitope will become in close contact with the mucosa and thereby effectively stimulate a
- 20 protective immune response in the form of the excretion of secretory antibodies of the IgA and IgG classes.

- Furthermore, the adhesion of the epitope-carrying cell will ensure that the cell is retained in the human or animal body for a period of time which is sufficient to obtain
- 25 the desired immune response. It is considered that a satisfactory immunization typically may be obtained if the cell is present in sufficient numbers in a particular body environment such as the intestinal tract for a period in the range of 15-30 days, depending on the nature and the activity of the epitope expressed from the cell.

- 30 As it will be understood from the above description of the gene coding for the cell function-limiting toxic polypeptide and the DNA sequence regulating its expression, the present invention may provide useful means of providing live vaccines based on recombinant organisms which are immunologically effective and which can be used

without the risk of undesired spreading of recombinant genes to the microflora of humans and animals or to the outer environment.

In accordance with the invention, a useful cell for the preparation of a live vaccine is one selected from a bacterial species which inherently contains an outer surface structure as mentioned above. Such species include as examples species of *Enterobacteriaceae* such as *Salmonella* and *E. coli* species, *Vibrionaceae* and *Pseudomonadaceae*. It will be understood that strains of such species which are particularly useful in the present invention as the basis of a live vaccine as defined above, are non-pathogenic strains or strains having a low pathogenicity.

The epitope expressed by a cell as defined above may be an epitope derived from any pathogenic organism or agent the obtainment of immunity against which is desirable. Such pathogens include viruses, bacteria and eukaryotic organisms such as fungi, yeast or protozoa.

In commercially important embodiments, the microbial cell comprising the gene coding for a cytotoxic polypeptide contains a nucleotide sequence coding for a pesticidally active gene product. In this context, the term "pesticidally active gene product" is used to denote a product which, when expressed in a cell being released to an environment where there is a need to reduce or eliminate the presence of pests that feed on plants, including insect pests, nematodes and vermins such as rodents or birds, is effective in respect of such pest control.

Such pests are currently controlled by the administration of toxic chemical pesticides to the infested environment, but recently various naturally occurring pesticidally active organisms including viruses, bacteria and fungi have been used as biological pest control products.

Prominent examples of such pesticidally active organisms include biotypes or strains of the species *Bacillus thuringiensis* that produce crystalline proteins being toxic to insects, in particular to caterpillars, and several viruses being pathogenic for insects in the larval stage or in the adult stage. However, the pesticidal effect of such organisms is frequently less satisfactory and there is a strong need in farming, forestry and horti-

culture to provide improved pesticidally active organisms. One approach to solving this problem is to construct genetically engineered organisms having an increased toxic effect or a better survival rate in the environment. In addition to pesticidally active compounds from *B. thuringiensis*, such compounds are produced by other microbial organisms including *Bacillus sphaericus*, fungal species, algal species and plants. In accordance with the invention, genes coding for such biopesticides can be inserted and expressed in the biologically contained cells of the invention.

To the extent such improved organisms are developed, their use in the environment will, as a consequence of current public concern of the potential risks involved in deliberate release of such toxic or pathogenic GEMs, only be approved by official environmental agencies if it can be demonstrated that the release does not lead to an undesired propagation or to an extended survival of such organisms in the environment to which they are applied.

The present invention clearly provides the means of limiting the survival in the environment of genetically engineered pesticidally active organisms. As it has been explained above, the rate of expression of the cytotoxic polypeptide can be regulated stochastically and thus the survival rate of pesticidally active cells may conveniently be adapted to any specific need. Also, the cell function-limiting effect of the toxic polypeptide may, in accordance with the present invention, be adjusted by selecting a first kind of polypeptide that has an appropriate cell function-limiting effect.

In another useful embodiment, the invention provides a cell in which the gene coding for a desired gene product is a sequence coding for a pollutant-degrading gene product. It is known that several xenobiotic compounds polluting the outer environment including soil and water can be degraded by microorganisms having an inherent capability of degrading these compounds. Obviously, the technology of genetic engineering provides means of providing improved organisms having an increased pollutant-degrading capacity or having the capacity to degrade a broad range of compounds, in particular hydrocarbons.

However, the public concern as mentioned above are also relevant in this context and accordingly, the present invention provides useful means of providing improved pollu-

tant-degrading microbial cells, the survival of which can be controlled by regulating the expression of the first kind of polypeptide as it is defined above. In particularly preferred embodiments, the cell contains a gene coding for a pollutant-degrading gene product, the expression of which is induced by the presence of a pollutant degradable  
5 by the cell.

In addition to the above desired gene products, the microbial cells according to the invention can express any desired gene product including pharmaceutically active products such as e.g. hormones, interleukines and antibiotically active peptides.

10

As mentioned above, the invention provides in a further aspect a method of confining an extrachromosomal replicon to a microbial cell population. Basically, the method comprises the steps of isolating or constructing a microbial cell containing a gene belonging to the *relE* gene family expressing a first kind of polypeptide that is toxic for  
15 the cell and introducing into the cell the extrachromosomal replicon to be confined, which replicon contains a gene coding for a second kind of polypeptide acting as an antitoxin for said first kind of polypeptide, and cultivating the cells under conditions where the genes coding for the first and the second kind of polypeptides are expressed, whereby a daughter cell that does not receive a copy of the extrachromo-  
20 somal replicon is killed by the first kind of polypeptide being expressed in the absence of expression of the second kind of polypeptide.

In preferred embodiments of such a method the cell population consists of cells that comprise a gene coding for a gene product of interest as defined above.

25

The above method of confining an extrachromosomal replicon is particularly useful when the replicon is a plasmid that naturally occurs in a host cell in a low copy number. Accordingly, the method is useful for confining a plasmid occurring in the microbial cells at a copy number which is in the range of 1-30 including the range of 1-10  
30 such as the range of 1-5.

Microbial cells to which a replicon can be confined in accordance with the invention include Gram-negative bacterial species such as species belonging to *Enterobacteriaceae*, *Hemophilus*, *Vibrionaceae* and *Pseudomonadaceae* and Gram-positive bacterial



species, fungal cells including yeast cells, animal cells including human cells and insect cells, and plant cells.

- In a still further aspect, the invention provides a method of post-segregationally stabilizing a plasmid in a microbial host cell population as described above. As it is mentioned above, the method comprises the steps of (i) inserting into the plasmid a gene coding for a first kind of polypeptide as defined herein and a gene coding for a second kind of polypeptide as also defined herein that is capable of being degraded in the host cell at a higher rate than that at which the first kind of polypeptide is degraded, (ii) cultivating the cell population under conditions where the genes coding for the first kind and second kind of polypeptides are expressed, whereby a daughter cell that does not receive at least one copy of the plasmid is killed as a result of the faster degradation of the second kind of polypeptide.
- 15 The invention also provides a recombinant microbial cell as defined above, comprising a gene coding for a first kind of polypeptide. Such a cell can be a bacterium of a Gram-negative bacterial species including *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp or it can be of a Gram-positive bacterial species such as a *Bacillus* species or lactic acid bacterial species, a fungal cell including a yeast cell, an animal cell including a human cell and an insect cell, and a plant cell.

- As also mentioned above, the invention pertains in another aspect to a method of limiting the survival of a cell population in a first or a second environment, which method comprises as the first step that the cells are transformed with a gene coding for a cytotoxic polypeptide, which gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide, the gene coding for the prophage P1 Doc polypeptide and a gene coding for a functionally equivalent polypeptide for anyone of said polypeptides.

In a specific embodiment of such a method, the survival of the cell population is limited in a first environment in which the gene is expressed whereby the cell population

is contained in said first environment. In another embodiment, the survival of the cell population is not limited when present in a first environment, which first environment could change to a second environment physically and/or chemically distinct from the first environment, in which first environment the gene whose expression results in the formation of a cytotoxically active polypeptide is not expressed, but the survival of which cell population is limited when transferred to a second environment or when present in a physically and/or chemically changed first environment, where the gene is expressed.

10 In a still further embodiment of the above method, the survival of a cell population is being limited by providing in the cells a gene coding for a cytotoxic polypeptide which is operably linked to a DNA sequence encoding an antitoxin repressor substance which can undergo a decay when said cells are released to the outer environment to an extent whereby the repressor substance is converted to a non-functional form, whereby  
15 as a result of said decay, the function of the cells of the population will be gradually limited.

In yet another aspect of the invention, there is provided a method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is  
20 naturally transferable to a second kind of cell, which method comprises as the first step providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc  
25 polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides.

In one specific embodiment of such a method the gene product which inhibits the expression of the expression of the gene coding for the polypeptide or the cell function-limiting effect of the polypeptide is selected from the *E. coli* relB polypeptide, the  
30 plasmid F CcdA polypeptide, the plasmid R1 PemI polypeptide, the plasmid RP4 ParD polypeptide, the prophage P1 Phd polypeptide and a functionally equivalent polypeptide of anyone of such polypeptides.

The invention also provides a method as defined above of stochastically limiting in an environment the survival of a cell population. Such a method is particularly useful in the containment of recombinant cells which are to be released to the outer environment or the animal or human body.

5

The invention will now be described in further details in the following examples and the drawings wherein

Fig. 1 illustrates *relB<sub>K-12</sub>::lacZ* and *relE<sub>K-12</sub>::lacZ* translational fusions. Shown are relevant parts of the *lacZ* reporter plasmids pKG4001 (carrying a *relB<sub>K-12</sub>::lacZ* fusion) and  
10 pKG4002 (carrying a *relE<sub>K-12</sub>::lacZ* fusion). Numbers to the right in the Figure indicate *lacZ* expression levels in Miller units. The low expression level of *relE::lacZ* in pKG4002 is, in part, due to the presence of an intact *relB* gene located on the plasmid. The *relB* gene product represses the *relBE* promoter c. 130-fold;

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Fig. 2 illustrates in vitro translation of *relBE<sub>P307</sub>*-carrying plasmids. Lane 1: pBR322; lane 2: pHA402 (pBR322-*relB*<sup>+</sup>); lane 3: pHA403 (pBR322-*relBE*<sup>+</sup>); lane 4: pBR322; lane 5: pHA100 (pBR322-E11 contains the P307 *relBE* genes in their natural context); lane 6: pKG325; lane 7: pHA110 (pBR325-*relB*<sup>+</sup>);

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Fig. 3 shows the structure of expression plasmid pNDM220. The plasmid is a mini-R1 vector whose copy number is amplifiable at 42°C due to the insertion of the temperature inducible  $\lambda$  P<sub>R</sub> promoter upstream of the replication control region. The plasmid also carries the *cl<sub>857</sub>* temperature-sensitive allele of the *cl* repressor. Genes shown are  
25 *copB* (copy number control), *repA* (initiation of replication), *parM* and *parR* (plasmid stability loci), *bla* ( $\beta$ -lactamase) and *lacP*. The plasmid contains the *LacI* regulated pA1/O4/O3 promoter upstream of a multiple cloning site that contains unique *BamHI* and *EcoRI* restriction sites. Thus genes inserted downstream of the promoter are inducible with IPTG;

30

Fig. 4 illustrates cell killing by *relE<sub>K-12</sub>* and anti-killing by *relB<sub>K-12</sub>*. Shown are optical density at 450 nm and viable counts as function of time for strains MC1000/pMG223 (*relE*<sup>+</sup>) (A, B), MC1000/pMG223/pMG2201 (*relB*<sup>-</sup> control plasmid) (C, D) and MC1000/pMG223/pMG2202 (*relB*<sup>+</sup> plasmid) (E, F). At time zero, transcription of *relE*

on plasmid pMG223 was induced by the addition of IPTG (1 mM). Filled symbols indicate that IPTG was added. As seen from (E) and (F), the presence of *relB* on a second plasmid counteracted *relE* mediated cell killing;

- 5 Fig. 5 shows the structure of expression plasmid pBAD33. The plasmid is a medium copy number pACYC-derived vector. The plasmid carries the arabinose inducible pBAD-promoter and the *araC* gene of *E. coli*. Thus upon addition of arabinose to pBAD33 containing cells, genes inserted downstream of pBAD are transcriptionally induced. Genes shown in the Figure are: pACYC-ori: origin of replication; CM(R): gene  
10 encoding chloramphenicol acetyl transferase; bla': truncated (nonfunctional) gene encoding  $\beta$ -lactamase; mRNA1 encodes AraC activator protein; pBAD: arabinose-inducible promoter;

Fig. 6 A/B illustrates cell killing by RelE<sub>P307</sub> and anti-killing by RelB<sub>P307</sub>. Shown are optical density at 450 nm (A,C) and viable counts (B, D) as a function of time for strains  
15 MC1000/pHA810/pBR322 (A, B) or MC1000/pHA810/pHA110 (carrying *relB*<sub>P307</sub>). At time zero, transcription of *relE*<sub>P307</sub> on plasmid pHA810 was induced by the addition of arabinose (0.02%). Filled symbols indicate that arabinose was added. As seen from (C) and (D), the presence of *relB*<sub>P307</sub> on a second plasmid counteracted *relE*<sub>P307</sub> mediated cell killing;  
20

Fig.7 shows maps of pHA705 and pHA715;

Fig. 8 illustrates OD<sub>450</sub> of MC1000/pHA-Sp2, MC1000/pHA705 and MC1000/pHA715 (+/- IPTG);  
25

Fig. 9 shows viable counts of MC1000/pHA-Sp2, MC1000/pHA705 and MC1000/pHA715 (+/- IPTG);

Fig. 10 is the DNA sequence of the *relBE*<sub>Sp2</sub> locus of *S. pneumoniae*;  
30

Fig. 11 is a map of pHA-Sp2;

Fig. 12 is a map of pHAG33;

Fig. 13 is a map of pHAG33-2;

Fig. 14 is a map of pHAG33-3;

5 Fig. 15 is a map of pHAG33-4;

Fig. 16 illustrates OD<sub>450</sub> of KT2440/pHAG33-2, KT2440/pHAG33-3 and  
KT2440/pHAG33-4 (+/- IPTG);

10 Fig. 17 shows viable counts of KT2440/pHAG33-2, KT2440/pHAG33-3 and  
KT2440/pHAG33-4 (+/- IPTG);

Fig. 18 is a map of pHA810;

15 Fig. 19 illustrates Glucose run-out, OD<sub>450</sub> of MC1000/pHA810;

Fig. 20 illustrates Glucose run-out, viable counts of MC1000/pHA810; and

Fig. 21 illustrates that RelE<sub>K12</sub>, RelE<sub>P307</sub> and RelE<sub>Mj</sub> inhibit translation *in vitro*

20

## EXAMPLES

### Materials and methods

25

#### **(i) Bacterial strains**

The *E. coli* K-12 strain MC1000 (Casadaban and Cohen, 1980) which contains a  
chromosomal copy of the *relBE* genes was used as the standard cloning strain and  
30 when a chromosomal copy of the *relB* operon was required. The *E. coli* K-12 strain  
JS115 (*leu*, *thy*, *thi*, *supE*,  $\Delta$ *relB*), which contains a deletion covering the entire *relB*  
operon was provided by Olle Karlström. The latter strain was used for the regulatory  
studies of *relBE*.

**(ii) Plasmids used**

Plasmid pOU253 is a mini-R1 based translational fusion vector carrying the *lacZ* gene of pNM482 (Minton, 1984). The fusion vector is segregationally stable due to the  
5 presence of the *parA* system of plasmid R1 (Dam and Gerdes, 1994).

Plasmid pNDM220 is a low copy-number mini-R1 expression vector carrying a multiple cloning site (mcs) placed between the LacI regulated pA1/O4/O3 promoter (Lanzer and Bujard, 1988) and two transcriptional terminators.

10

pNDM220 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12157.

15 Plasmid pBD2430 (+388 - +1899) is a pUC18 derivative carrying the complete *relBE* operon and gene *IV* located downstream of *relF* (Olle Karlström, unpublished). The relevant *E. coli* DNA present in pBD2430 is shown in Table 1.1 below.

pBD2430 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-  
20 Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12161.

**(iii) Plasmids constructed**

25 pKG4001: pBD2430 was digested with *EcoRI* and *XhoI* and the fragment carrying the *relB* promoter (Table 1.1) was inserted into pOU253 producing an in-frame translational fusion between *relB*<sub>K-12</sub> and *lacZ*. Thus, pKG4001 carries a *relB*<sub>K-12</sub>::*lacZ* translational fusion.

30 pOU253 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12158.

pKG4002: pBD2430 was digested with *EcoRI* and *Bst*1107I and the resulting fragment was inserted into pOU253 producing an in frame translational fusion between *relE*<sub>K-12</sub> and *lacZ*. Thus pKG4002 carries an intact *relB*<sub>K-12</sub> gene and a *relE*<sub>K-12</sub>::*lacZ* translational fusion.

5

pMG223: *relE*<sub>K-12</sub> was amplified by PCR on pBD2430 with primers *relE*1B (5'-CCCCGGATCCATAAGGAGTTTTATAAATGGCGTATTTTCTGGATTTTGACG, SEQ ID NO:1) containing the *parA* Shine & Dalgarno (Gerdes and Molin, 1986) and *relE*2 (5'-CCCCCCTCGAGGTCGACTCAGAGAATGCGTTTGACCGC-3', SEQ ID NO:2). The resulting *relE*<sub>K-12</sub> carrying fragment was inserted into pNDM220 using the *Bam*HI and *Sal*I restriction sites. Plasmid pMG223 expresses RelE<sub>K-12</sub> upon addition of IPTG.

10

pMG2201: this plasmid contains the *EcoRI-Eco*47III fragment from pBD2430 inserted between the *EcoRI* and *Sca*I sites of pBR322. Plasmid pMG2201 carries the *relB*<sub>K-12</sub> promoter and the 5' part of the *relB*<sub>K-12</sub> gene.

15

pMG2202: pBD2430 was digested with *EcoRI* and *Bst*1107I and the *relB*<sub>K-12</sub>-carrying fragment was inserted into pBR322 *EcoRI-Sca*I. The resulting plasmid carries the *relB* promoter and *relB*<sub>K-12</sub>.

20

pHA100: Plasmid pNZ945 is a pBS(+) derivative that carries a 4.3 kb *EcoRI* fragment from plasmid P307. This fragment encodes the RepFIB replicon and the *relBE* genes of P307 (Saul et al., 1989). The 4.3 kb *EcoRI* fragment (designated E11) of pNZ945 was purified and restricted with *Pst*I. The resulting 2.2 kb *EcoRI-Pst*I fragment was inserted into pBR322 restricted also with *EcoRI* and *Pst*I. The pBR322-derived plasmid carrying the 2.2 kb *EcoRI-Pst*I fragment was designated pHA100. Plasmid pHA100 codes for the entire *relBE* system from P307.

25

pNZ945 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12160.

30

pHA110: The 2.2 kb *EcoRI-Pst*I fragment of pHA100 was purified and digested with *Apo*I (*EcoRI* isoschizomer). The resulting *EcoRI-Apo*I DNA fragment (+1 to +1122)

was inserted into the *EcoRI* site of pKG325 which was constructed as follows: Plasmid pBR325 was restricted with *PstI*, which has a unique recognition site in the plasmid. The resulting vector DNA fragment was made blunt ended with T4 DNA polymerase according to the manufacturer's instructions, and religated. Transformants  
5 that were resistant to chloramphenicol and tetracycline, but sensitive to ampicillin were selected. Thus, pKG325 is a TcR, CmIR and ApS derivative of pBR325.

pKG325 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-  
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession  
10 No. DSM 12159.

Plasmid pHA110 contains the *relB* promoter (*preIB<sub>P307</sub>*) and gene *relB<sub>P307</sub>*.

pHA205: Plasmid pHA205 is a derivative of the low copy-number mini-R1 expression  
15 vector pNDM220 that contains the *relB* gene from P307. The PCR fragment generated from pNZ945 using primers RelB-P307/1: 5'-

CCCCCGGATCCCAGTCTTGAAAGGTGGC-3' (SEQ ID NO: 3) and RelB-P307/2: 5'-  
CCCCCGAATTCTCATAGGTATTTATCCAG-3' (SEQ ID NO:4) was restricted with  
*Bam*HI and *Eco*RI and inserted downstream of the pA1/04/03 promoter of pNDM220.

20 pHA210: Gene *relE<sub>P307</sub>* was PCR-amplified from pNZ945 with the primers: *relE*-p307/3 (5'-CCCCGGATCCAGATCTGGATAAATACC, SEQ ID NO:5) and *relE*-P307/2 (5'-  
CCCCCGAATTCGTAACCTTTCTGTGTTTATTGC, SEQ ID NO:6). The resulting PCR DNA fragment was restricted with *Bam*HI and *Eco*RI and inserted into pNDM220 also restricted with *Bam*HI and *Eco*RI. Plasmid pHA210 (+ 1089 to + 1417) is thus a mini-R1  
25 derivative carrying a pA1/04/03::*relE<sub>P307</sub>* gene fusion which renders *relE<sub>P307</sub>* inducible with IPTG.

pHA215: Genes *relBE<sub>P307</sub>* were PCR-amplified from pNZ945 with the primers RelB-P307/1 (5'-CCCCCGGATCCAGTCTTGAAAGGTGGC, SEQ ID NO:3) and *relE*-P307/2  
30 (5'-CCCCCGAATTCGTAACCTTTCTGTGTTTATTGC, SEQ ID NO:6). The resulting PCR-generated DNA fragment was restricted with *Bam*HI and *Eco*RI and inserted into pNDM220 also restricted with *Bam*HI and *Eco*RI. Plasmid pHA215 (+ 840 to + 1417) is thus a mini-R1 derivative carrying a pA1/04/03::*relBE<sub>P307</sub>* gene fusion rendering the *relBE<sub>P307</sub>* genes inducible with IPTG.



pHA402: A *Pst*I-*Aat*II fragment from plasmid pHA205, which carries *lac*<sup>R</sup> and the pA1/O4/O3::*relB*<sub>P307</sub> gene fusion was inserted into pBR322 also restricted with *Pst*I and *Aat*II. Thus, the high copy-number plasmid pHA402 contains a *relB*<sub>P307</sub> gene which is inducible with IPTG.

5

pHA403: A *Pst*I-*Aat*II fragment from plasmid pHA215, which carries *lac*<sup>R</sup> and the pA1/O4/O3::*relBE*<sub>P307</sub> gene fusion was inserted into pBR322 also restricted with *Pst*I and *Aat*II. Thus, the high copy-number plasmid pHA403 contains the *relBE*<sub>P307</sub> genes which can be conditionally induced by the addition of IPTG.

10

pHA810: A DNA fragment encoding *relE*<sub>P307</sub> was generated by PCR using primers *relE*-P307/4 (5'-CCCCCGAGCTCAGATCTGGATAAATACC, SEQ ID NO:7) and *relE*-P307/5 (5'-CCCCCGCATGCGTAACTTTCTGTGTTTATTGC, SEQ ID NO:8). The fragment was digested with *Sac*I + *Sph*I and inserted into the expression plasmid pBAD33 also digested with *Sac*I + *Sph*I. The resulting plasmid, pHA810 (+ 1089 - + 1417), contains the pBAD::*relE*<sub>P307</sub> gene fusion that renders *relE*<sub>P307</sub> inducible with arabinose.

15

An overview of the bacterial strains and plasmids used herein is shown in Table O.1 below.

20

25

30

Table O.1. Bacterial strains and plasmids

Strains	genotypes	Reference/Source		
5 MC1000	<i>Δlac leu ara</i>	Casadaban & Cohen, 1980		
JS115	<i>ΔrelB leu thy thi supE</i>	J. P. Bouche, unpublished		
Plasmids	Replicon	Resistance <sup>a)</sup>	<i>relBE</i> co-ordinates <sup>b)</sup>	Reference/Source
10 pOU253	mini-R1	Ap <sup>R</sup>	none	lab. collection
pBAD33	pACYC	Cml <sup>R</sup>	none	Guzman et al., 1995
pNDM220	mini-R1	Ap <sup>R</sup>	none	Gotfredsen & Gerdes, 1998
pBR322	ColEI	Ap <sup>R</sup> , Tc <sup>R</sup>	none	Bolivar et al., 1978
pKG325	pBR325	Tc <sup>R</sup>	none	lab. collection
15 pBD2430	pUC	Ap <sup>R</sup>	+388 - +1899	Olle Karlström collection
pNZ945	pUC	Ap <sup>R</sup>	+1 - +4298	Saul et al., 1989
pKG4001	mini-R1	Ap <sup>R</sup>	+388 - +596	Gotfredsen & Gerdes, 1998
KG4002	mini-R1	Ap <sup>R</sup>	+388 - +921	Gotfredsen & Gerdes, 1998
pHA100	pBR322	Tc <sup>R</sup>	+1 - +2198	Grønlund & Gerdes, 1998
20 pHA110	pBR325	Tc <sup>R</sup>	+1 - +1122	Grønlund & Gerdes, 1998
pHA205	mini-R1	Ap <sup>R</sup>	+840 - +1111	Grønlund & Gerdes, 1998
pHA210	mini-R1	Ap <sup>R</sup>	+1089 - +1417	Grønlund & Gerdes, 1998
pHA215	mini-R1	Ap <sup>R</sup>	+840 - +1417	Grønlund & Gerdes, 1998
pHA402	pBR322	Tc <sup>R</sup>	+840 - +1111	Grønlund & Gerdes, 1998
25 pHA403	pBR322	Tc <sup>R</sup>	+840 - +1417	Grønlund & Gerdes, 1998
pHA810	pACYC	Cml <sup>R</sup>	+1089 - +1417	Grønlund & Gerdes, 1998
pMG223	mini-R1	Ap <sup>R</sup>	+733 - +1020	Gotfredsen & Gerdes, 1998
pMG2201	pBR322	Tc <sup>R</sup>	+388 - +597	Gotfredsen & Gerdes, 1998
pMG2202	pBR322	Tc <sup>R</sup>	+388 - +921	Gotfredsen & Gerdes, 1998

a) Tc<sup>R</sup>, tetracycline resistance; Ap<sup>R</sup>, ampicillin resistance; Cml<sup>R</sup>, chloramphenicol resistance.

b) Co-ordinates refer to Table 1.1 (*relBE*<sub>K-12</sub>, pMG-plasmids) or Table 1.2 (*relBE*<sub>p307</sub>, pHA-plasmids)

#### (iv) Growth media and antibiotics

35

The growth medium was LB medium (Bertani, 1951) or A + B minimal medium (Clark and Maaløe, 1967) supplemented with 0.2% glucose and 1% casamino acids. For growth on solid media, LA-plates were used. LA is LB containing 15 g agar per litre. All media were supplemented with 50 μg/ml thymine for growth of the strain

JS11507-05-99 $\Delta re/BEF_{K-12}$ . Antibiotics were added at the following concentrations: ampicillin, 30  $\mu\text{g/ml}$ , and tetracycline, 10  $\mu\text{g/ml}$ . When indicator plates were used X-gal (5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) was added to a final concentration of 40  $\mu\text{g/ml}$ .

5

**(v) Conditions of cell growth.**

Cells were diluted in LB + antibiotics from an overnight culture to an  $\text{OD}_{450}$  of 0.005. The cultures were then grown at 37°C until an  $\text{OD}_{450}$  of 0.4 and then diluted to an  
10  $\text{OD}_{450}$  of 0.01 in 37°C LB containing 1 mM IPTG and antibiotics. Samples for  $\text{OD}_{450}$  measurements and viable counts were taken at the time points indicated. Viable counts were made by plating dilutions of the cultures onto LA plates containing the proper antibiotics.

15 **(vi) Coupled *in vitro* transcription and translation.**

The reactions were performed using the *E. coli* S30 Extract System For Circular DNA as described by the supplier (Promega Corp.). 4  $\mu\text{g}$  of DNA was used in all reactions. The reactions were run on a 16% Tricine-SDS-PAGE gel essentially as described by  
20 Schägger and von Jagow (1987).

**(vii)  $\beta$ -galactosidase assays.**

$\beta$ -galactosidase assays were performed essentially as described by Miller (1972).  
25

**(viii) Homology search.**

BLAST searches were performed at the GENESTREAM BLAST network server CRBM Montpellier, France. Standard conditions were used except that the blosum 80 matrix  
30 was used.

## EXAMPLE 1

**The occurrence of *relBE* operons in bacteria and *Archae***5 **1.1. Nucleotide sequence of the *relBE* operon of *E. coli* K-12**

The DNA sequence of the *relBE* operon from *E. coli* K-12 is shown in Table 1.1. In this Table the transcriptional start site of the *relBE* mRNA is indicated with two asterisks (heterogeneity). IR indicates inverted repeats in the promoter and terminator regions.  
 10 Start codons and stop codons are shown in bold. The transcriptional termination point (*ttp*) of the *relBE* mRNA is also indicated with a vertical arrow. The DNA sequence is from Bech et al., 1985.

By visual inspection of the *relB*<sub>K-12</sub> and *relE*<sub>K-12</sub> genes there was found striking similarity  
 15 with the so-called "proteic plasmid stabilization systems" as described by Jensen and Gerdes (1995). First, *relE*<sub>K-12</sub> codes for a very basic protein (RelE<sub>K-12</sub>; pI = 9.7) of 95 amino acids (aa), and *relB*<sub>K-12</sub> codes for a very acidic protein (RelB<sub>K-12</sub>; pI = 4.8) of 79 aa.

20 The sequences of proteins RelB<sub>K-12</sub> and RelE<sub>K-12</sub> are shown in Tables 1.5 and 1.6, respectively. These Tables show multiple sequence alignments of the RelB and RelE gene families. Conserved amino acids at a given position are shown with shading as follows: two amino acids are considered conserved if they both belong to one of the following groups: group 1: D and N; group 2: E and Q; group 3: S and T; group 4: K  
 25 and R; group 5: F, Y and W; group 6: L, I, V and M. Light grey shading indicates 60-80% conservation, dark grey indicates 80-99% conservation and black indicates 100% conservation. Note in Table 1.6 the fully conserved glycine at position 69 (G in consensus line) and the fully conserved arginine at position 79 (R in consensus line). The entrez database accession numbers of the protein sequences are given in Tables  
 30 1.3 and 1.4.

The *relB*<sub>K-12</sub> and *relE*<sub>K-12</sub> genes are co-transcribed with a third gene, *relF* (also denoted *orf-3* or *hokC*), which is homologous to the *hok* gene from plasmid R1 (Gerdes et al., 1986). The start site (i.e. the 5'-end) of the *relBE* mRNA was determined to be 31 nu-

cleotides upstream of the *relB*<sub>K-12</sub> AUG start-codon (Bech et al., 1985) and was confirmed (M. Gotfredsen and K. Gerdes, 1998). Inverted arrows in the *relBE* promoter region (Table 1.1) indicate putative binding sites for regulators of transcription (i.e. the RelB<sub>K-12</sub> and RelE<sub>K-12</sub> proteins themselves).

5

The properties described above suggested that RelE could be a cytotoxin and that RelB could be an antitoxin which counteracts the toxicity elicited by RelE.

Table 1.1. DNA sequence of the *relBE* operon from *E. coli* K-12 (SEQ ID NO:9)

10

```

      1  CTTAATTTC A GGCCCCATCG GATCACACAT GGAGAGTTTT TATGAATAAC
     51  CCCGCTCTGTC TTGATGACTG GTTGATTGGC TTTAAAAGCT TGTTGACAGG
    101  GGTAAACGTT CGGCAATAAT TTTCTGCCGC ATGCGGGTGT TGCATAAAAC
    151  GTGTTACGTT CCTTTATCGA CAGGTCAGGT CACCGCTCAC CCGCCGACGA
    201  GAAAGCAACA CTGACATGCT AAAGCAAAAA ATAGATGAAT AAGTTGAGTT

    251  GTGCATATGT AGCCTGACCG TCACAAAGTA TATGGTGTCT GTACCAGTAA

    301  GATGATGGCC GGACTCTTTA AAAACGAGCT GACCTGCACA ATACAGGATG

    351  GACTTAGCAA TGGCTGCTCC TGGCACAAAG CGGACAGTGA TCACCGTTCT

    401  TACGACTACT TTCTGACTTC CTTCGTGACT TGCCCTAAGC ATGTTGTAGT

    451  GCGATACTTG TAATGACATT TGTAATTACA AGAGGTGTAA GACATGGGTA
          ---- -->IR<--- ----
    501  GCATTAACCT GCGTATTGAC GATGAACTTA AAGCGCGTTC TTACGCCGCG

    551  CTTGAAAAAA TGGGTGTAAC TCCTTCTGAA GCGCTTCGTC TCATGCTCGA

    601  GTATATCGCT GACAATGAAC GCTTGCCGTT CAAACAGACA CTCCTGAGTG

    651  ATGAAGATGC TGAAC TTGTG GAGATAGTGA AAGAACGGCT TCGTAATCCT

          End relB      Start relE
    701  AAGCCAGTAC GTGTGACGCT GGATTGAACTC TGATGGCGTA TTTTCTGGAT

    751  TTTGACGAGC GGGCACTAAA GGAATGGCGA AAGCTGGGCT CGACGGTACG

    801  TGAACAGTTG AAAAAGAAGC TGGTTGAAGT ACTTGAGTCA CCCC GGATTG

    851  AAGCAAACAA GCTCCGTGGT ATGCCTGATT GTTACAAGAT TAAGCTCCGG

    901  TCTTCAGGCT ATCGCCTTGT ATACCAGGTT ATAGACGAGA AAGTTGTCTG

    951  TTTCGTGATT TCTGTTGGGA AAAGAGAACG CTCGGAAGTA TATAGCGAGG

    1001  CGGTCAAACG CATTCTCTGA ACCAAAGCAT GACATCTCTG TTTCGCACCG

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                                     Start hokC (relF)
1051  AAGGTGACAC TTCTGCTTTG CGTTGACAGG AGAAGCAGGC TATGAAGCAG
5
1101  CAAAAGGCGA TGTTAATCGC CCTGATCGTC ATCTGTTTAA CCGTCATAGT
1151  GACGGCACTG GTAACGAGGA AAGACCTCTG CGAGGTACGA ATCCGAACCG
                                     End hokC
1201  ACCAGACGGA GGTCGCTGTC TTCACAGCTT ACGAACCTGA GGAGTAAGAG
10
1251  ACCCGGCGGG GGAGAAATCC CTCGCCACCT CTGATGTGGC AGGCATCCTC
1301  AACGCACCCG CACTTAACCC GCTTCGGCGG GTTTTTGTTT TTATTTTCAA
15
      ---- -- IR ---- - ttp
1351  CGCGTTTGAA GTTCTGGACG GTGCCGGAAT AGAATCAAAA ATACTTAAGT
      (data base accession number X02405)
```

Table 1.3. *relE* homologues from Gram-positive and Gram-negative bacteria and *Archae*

5	Bacterial species	entrez accession	gene <sup>a)</sup>	Number of aa	MW (kD)	pI
<b>Gram-negative bacteria:</b>						
10	<i>E. coli</i> K-12	132284	<i>relE</i> <sub>K-12</sub>	95	11.2	9.7
	<i>E. coli</i> K-12	984581	<i>relE</i> <sub>SOS</sub> <sup>b)</sup>	92	10.8	9.5
	<i>E. coli</i> plasmid P307	516611	<i>relE</i> <sub>P307</sub>	95	11.2	9.9
15	<i>H. influenzae</i>	1175293	<i>relE</i> <sub>Hi</sub>	102	11.9	6.7
	<i>V. cholera</i>	396846	<i>relE</i> <sub>Vc</sub>	96	11.2	9.9
	<i>H. pylori</i>	2314031	<i>relE</i> <sub>Hp</sub>	88	10.4	7.9
20	<i>Synechosystis</i>	1653777	<i>relE</i> <sub>Sy</sub>	120	13.7	7.9
<b>Gram-positive bacteria:</b>						
25	<i>B. thuringiensis</i>	520407	<i>relE</i> <sub>Bt</sub>	74	8.6	9.7
	<i>M. tuberculosis</i> #1	2612811	<i>relE</i> <sub>Mt1</sub>	87	10.2	11.0
	<i>M. tuberculosis</i> #2	2695832	<i>relE</i> <sub>Mt2</sub>	97	11.1	9.5
<b>Archae:</b>						
30	<i>M. jannaschii</i> #1	1498833	<i>relE</i> <sub>Mj1</sub>	90	11.0	10.2
	<i>M. jannaschii</i> #2	1499953	<i>relE</i> <sub>Mj2</sub> (*)	88	10.6	10.0
35	<i>M. jannaschii</i> #3	1591583	<i>relE</i> <sub>Mj3</sub> (*)	91	11.1	10.1
40	<i>A. fulgidus</i> #1	2648176	<i>relE</i> <sub>Af1</sub>	87	10.6	10.3
	<i>A. fulgidus</i> #2	2649499	<i>relE</i> <sub>Af2</sub>	92	11.0	9.9
	<i>A. fulgidus</i> #3	2649496	<i>relE</i> <sub>Af3</sub>	85	10.0	10.0
	<i>A. fulgidus</i> #4	2649514	<i>relE</i> <sub>Af4</sub>	86	10.2	9.9

45 a) *relE* homologues marked with (\*) are not located adjacent to a *relB* partner

b) The *relBE*<sub>SOS</sub> system of *E. coli* K-12 contains a LexA binding-site in the promoter region (Lewis et al., 1994)

Table 1.4. *relB* homologues from Gram-positive and Gram-negative bacteria and *Archae*

5	Bacterial species	entrez accession	gene <sup>a)</sup>	Number of aa	MW (kD)	pI
<b>Gram-negative bacteria:</b>						
	<i>E. coli</i> K-12	132283	<i>relB</i> <sub>K-12</sub>	79	9.1	4.8
10	<i>E. coli</i> K-12	984582	<i>relB</i> <sub>SOS</sub> <sup>b)</sup>	86	9.4	5.2
	<i>E. coli</i> K-12	984588	<i>relB</i> <sub>K-12,2</sub> (*)	97	11.2	5.5
15	<i>E. coli</i> plasmid P307	516610	<i>relB</i> <sub>P307</sub>	83	9.2	4.4
	<i>S. typhimurium</i>	731639	<i>relB</i> <sub>St</sub> (*)	68	7.6	5.3
	<i>H. influenzae</i>	1573712	<i>relB</i> <sub>Hi</sub>	98	11.0	4.7
20	<i>V. cholera</i>	396847	<i>relB</i> <sub>Vc</sub>	82	8.9	4.4
	<i>H. pylori</i>	2314037	<i>relB</i> <sub>Hp</sub>	95	11.4	9.8
25	<i>Synechosystis</i>	1653776	<i>relB</i> <sub>Sy</sub>	86	9.9	4.7
<b>Gram-positive bacteria:</b>						
	<i>B. thuringiensis</i>	520406	<i>relB</i> <sub>Bt</sub>	85	10.1	4.5
30	<i>M. tuberculosis</i> #1	2612810	<i>relB</i> <sub>Mt1</sub>	93	10.2	4.6
	<i>M. tuberculosis</i> #2	2695833	<i>relB</i> <sub>Mt2</sub>	89	9.8	5.1
<b>Archae:</b>						
35	<i>M. jannaschii</i> #1	1498832	<i>relB</i> <sub>Mj1</sub>	82	9.6	4.5
	<i>A. fulgidus</i> #1	2648190	<i>relB</i> <sub>Af1</sub>	65	7.8	4.8
40	<i>A. fulgidus</i> #2	2649516	<i>relB</i> <sub>Af2</sub>	62	7.4	4.3
	<i>A. fulgidus</i> #3	2649510	<i>relB</i> <sub>Af3</sub>	72	8.5	4.5
	<i>A. fulgidus</i> #4	269513	<i>relB</i> <sub>Af4</sub>	57	6.7	4.1

a) *relB* homologues marked with (\*) are not located adjacent to a *relE* partner.

b) The *relBESOS* system of *E. coli* K-12 contains a LexA binding-site in the promoter region (Lewis et al., 1994).



[illegible]

Table 1.6. Alignment of *relB* homologues from Gram-positive and Gram-negative bacteria and *Archae* (SEQ ID NOS:27-43)

55	relB-SOS	-----MAANAFVRRARDEDLKNQAADVLAGMGLTISDVRIT-LTKVAREKALPFDL--REPNO LTIQSIKN-----SE : 66
50	relB-VC	-----MTTRILADAASTTTFKXANPMKVATSAFGAPVAVIN-----RNEPAFYCVP--ASTYFIMMDKLED-----LE : 61
45	relB	-----MPNIIISDTSAS--SE--KNPMKMATVSAGDGFVAIIN-----RNQPAFYCVP--AELYKMLDALDD-----QE : 61
40	relB-Mt1	MRILPISITIKGNEFDASSTQDQITITKNGAPAAVVGAD-EWESLQETLYWLAQ--PGIRSSIAEADAD-----IA : 72
35	relB-Mt2	MAVPLIGEVRNRSEYAEWELTHERITITRHGPAAVISAD-DLASIEETLEVLRT--PGASSAIREGLAD-----VA : 72
30	relB-K12_2	-----MHRILAEKSVNTEIRKNPAKYFID--QPVAVIS-----NNRPAGYLLS--ASAFALMDMLAEQEKKPIKARFRPSAARLE : 74
25	relB-St	-----MFMRVTNYSSEARQNLAEVLESAVTGVPVITR-----RGHKSAVIIS--AEFFRYQTARMD-----D : 56
20	relB-coli	-----MGSNLRDDELKARSYAALCKMGVTPSEALR-----LMLEYIADNE--RLPFKQTLSDSD-----AE : 57
15	relB-HI	-----MALTNSS--SFRTVETKTKLEAYQVIEQYGLTPSQFNMFLAQIAKTRSIIPVDLNLVLRPNKETLAIDELD-----SGNAES : 75
10	relB-MJ	-----MLNINKEAQIETENELKLRDEISERIEKLK-----LLKLKALAIPE--EEFEDDYEEIIED-----VK : 61
5	relB-Af1	-----MNEALREIYS--KKIREKIEQLEELIIPAENVSEELLEIRKLKEESLKGHVWD--LKRRLGV-----LE : 65
	relB-Af3	-----MKVLLDI--EDIENFIRQLEKRRGELEELKDEILIFS-----DAEFIDSIQIGSLDLE--GRSKVCSN-----LE : 63
	relB-Af2	-----MEVLIQISKDETEFEHIERKFKEVLIKALMEITPYVSD-----EQEEDIKIAGKPDEYEGEFEEWHGK-----LE : 62
	relB-Af4	-----MDIQVAKQAR--RELRBELPSILKEVILSTIPPD-----EPEADEKQFVDEEIN--DDYVKFDE-----LE : 57
	relB-Hp	-----MPNTTKDYTKYS--RQLFSFLNSIKTKQKRALEKLKEIQAKQRIKKALQFKALNLTENGTYTIEERE-----ILARAK : 74
	relB-synec	-----MMRAFEVATVKDSKQLLSDSLHWNTSRVKVILESD-----ELASKGSEFDP--DDTPV--EIKVSLRK-----AL : 65
	relB-BT	-----MAIRKDEAYRL--DH--DQDQDEKAAFDLEFLVQSRRRKP-----KEWEKIDMADPDHPEPLSTQLELEQLNS-----E : 65
55	relB-SOS	100 : AGIDVHKAKDADDLFDKLG I-- : 86
50	relB-VC	100 : LLAIAKERLSSEDSVSVNIDDL-- : 82
45	relB	100 : LVKLVAERSNQPLHDVLDKYL- : 83
40	relB-Mt1	100 : SGRTYGEDEIRAEEFGVPRPH-- : 93
35	relB-Mt2	100 : AGRFVSNDIEIRNRYTAR-- : 89
30	relB-K12_2	100 : EITRAEQYLNDMTDDDFNDFKE : 97
25	relB-St	100 : EFAAIMAVHGNE-- : 68
20	relB-coli	100 : LVEIVKERLRNPKPVRVTLDEL- : 79
15	relB-HI	100 : FFIEASENYSAAEEFTKRILNGGQ : 98
10	relB-MJ	100 : KSLDKKETVPAEEALKELGLL-- : 82
5	relB-Af1	100 : ----- : -
	relB-Af3	100 : EVKCLFEDI----- : 72
	relB-Af2	100 : ----- : -
	relB-Af4	100 : ----- : -
	relB-Hp	100 : DTKNRLCFKSIEDFKKHCENL-- : 95
	relB-synec	100 : EYKQKGKRIPIVENWEGIDVE-- : 86
	relB-BT	100 : EGYVSGEDAKREFGLQIDLP-- : 85

### 1.2. Nucleotide sequence of the *relBE* operon of plasmid P307

By database searching it was found that the *E. coli* plasmid P307 codes for a gene system which exhibits both structural and sequence similarity with the *E. coli relBE* genes described above.

The DNA sequence of the *relBE*<sub>P307</sub> genes is shown in Table 1.2. The transcriptional start site of the *relBE* mRNA is indicated with an asterisk, and the -10 and -35 sequence elements of the *relBE* promoter are underlined. The Shine & Dalgarno sequence of the *relB* and *relE* genes are doubly underlined. The DNA sequence is from Saul et al., 1989.

Again, *relE*<sub>P307</sub> codes for a very basic protein of 95 aa (pI = 9.9), and *relB*<sub>P307</sub> codes for a very acidic protein of 83 aa (pI = 4.4), see Tables 1.3 and 1.4. The protein sequences of RelE<sub>P307</sub> and RelB<sub>P307</sub> are also shown in Tables 1.5 and 1.6, respectively. The start site (i.e. the 5'-end) of the *relBE*<sub>P307</sub> mRNA was determined to be located 27 nucleotides upstream of the *relB*<sub>P307</sub> AUG start codon. Inverted arrows in the *relBE*<sub>P307</sub> promoter region (Table 1.2) indicate putative binding sites for regulators of transcription (i.e. the RelB<sub>P307</sub> and RelE<sub>P307</sub> proteins).

Table 1.2. DNA sequence of the *relBE* operon from the *E. coli* plasmid P307 (SEQ ID NO:44)

5	301	GAGTATCATA	TTAGGATACG	GGTGGGTGAC	GCCACCTCT	GGCATAGAAC
	351	GGACATTCAT	TGATGCCATG	CCAGAATGGA	CGTTCAGGTT	ATTCCGTCCA
	401	GTTCTGCTGG	CAACGCGAGA	TCTCCCCTGG	TATAGTGATG	CCACAGCAAA
10	451	GCGCTCAAAC	AGGGATAATA	TGATGGAAAT	CAAGGCTCAA	CAGTTTTGTC
	501	ACATCAACGG	GGCGGCAAGT	CCTTACTGAC	AACGGACAAC	AAGGTATGGG
	551	CGGCGTGGCG	GGTATCGGTT	CCACGACTGA	AAAGCATCAG	GGGCGCGTGG
15	601	CGGAAGCGAT	TTTTGCGAAC	TGCGCGGAAC	TGGATAACGA	CCAGCTTAAC
	651	GAGATCATCG	AGTGGGTTCG	GCTCTATCAG	CGCTGAATGC	CACTATCAGG
	701	CTGCGCAAGC	GGCCTTTTTT	ACGCCCTTG	TTTAATTCCC	GCACTACCTG
20						-35
	751	GACG TTCAGG	TGATTCTGTC	CATCTGTACA	AAAAACAATA	AAAGACTTGT
25			-10	*→ <i>relBE</i> <sub>P307</sub> mRNA		
	801	<u>TAACAGGTCA</u>	TGTAAGGAGT	<u>ATCTTTGAGA</u>	CTGGTTAAAC	AGTCTT <u>GAAA</u>
30		SD	start <i>relB</i>			
	851	<u>GGTGGCCTAT</u>	GCCTAACATT	ATTCTCAGTG	ATACAAGCGC	CAGTGTCAGC
35	901	GAGCTGAAGA	AAAACCCGAT	GGCGACAGTC	AGCGCCGGTG	ATGGTTTCCC
	951	GGTCGCTATC	CTGAACCGTA	ATCAGCCTGC	TTTCTACTGT	GTACCCGCAG
	1001	AGCTGTACGA	AAAGATGCTT	GATGCCCTAG	ACGATCAGGA	GTTGGTTAAA
40					SD	
	1051	CTGGTAGCCG	AACGCAGCAA	CCAACCGCTG	CATGATGTAG	ATCT <u>GGATAA</u>
45		end <i>relB</i> /start <i>relE</i>				
	1101	ATACCT <b>ATGA</b>	GGTATCAGGT	AAAATTCAGG	GAAGATGCGC	TGAAAGAGTG
	1151	GCAAAACTG	GACAAGGCTA	TTCAGCAACA	GTTTGCGAAA	AAGCTAAAAA
50	1201	AGTGCTGTGA	CAATCCGCAT	ATTCCTTCCG	CAAACTGCG	TGGGATAAAG
	1251	GACTGCTACA	AAATAAAATT	ACGTGCGTCA	GGTTTTCGCC	TGGTCTATCA
	1301	GGTGATTGAC	GAACAATTAA	TTATCGCTGT	TGTAGCTGTG	GGTAAACGTG
						end <i>relE</i>
	1351	AGCGCAGTGA	CGTTTATAAT	CTTGCCAGCG	AAAGAATGAG	<b>ATAAAAGCAA</b>

5

1401 TAAACACAGA AAGTTACTCT GCGTTATGG GGTAATGCAA AGTATGAGTC  
1451 GTAGAGGGAA TTGCCTGGAT AATTCGCCGA TGGAAAGAGT CTTTCGCAGC  
1501 CTTAAAAGTG AATGGCTTCC GAAAGGTGGT TATGGTGATT TTAGCCATGC  
(database accession number M26308)

### 1.3. Nucleotide sequence and proteins of a *relBE* homologous operon from *Bacillus thuringiensis*

Using BLAST database searching (Altschul et al., 1990) it was found that transposon  
5 Tn5401 from the Gram-positive organism *B. thuringiensis* contains, in one end or  
asymmetrically located, a two-component system which exhibits both structural and  
sequence similarity with the above described *relBE* systems from *E. coli*. This homol-  
ogy is surprising given that it has not previously been described that *relBE*-like genes  
are found in organisms other than *E. coli*.

10

The nucleotide sequence of the *relBE* operon from Tn5401 is shown in Table 1.7. In  
this Table the transcriptional start-site of the *relBE* mRNA is indicated with an asterisk  
(Baum, 1994). IR indicates inverted repeats in the *relBE<sub>Bt</sub>* promoter region. Start co-  
dons and stop codons are shown in bold. The Shine & Dalgarno sequence of the *relB<sub>Bt</sub>*  
15 gene is doubly underlined. The DNA sequence is from Baum et al., 1989.

The *relE<sub>Bt</sub>* gene codes for a very basic protein of 74 aa (pI = 10.6) and the *relB<sub>Bt</sub>* gene  
codes for an acidic protein of 87 aa (pI = 4.4). The protein sequences of RelE<sub>Bt</sub> and  
RelB<sub>Bt</sub> are aligned with the other RelE and RelB homologues in Tables 1.5 and 1.6, re-  
20 spectively. The modular, structural and physico-chemical similarities between the *B.*  
*thuringiensis* system and the *E. coli* systems suggested that the genes may exert simi-  
lar functions in very different bacteria.

Table 1.7. DNA sequence of the *relBE* operon from the Gram-positive organism *B. thuringiensis* (SEQ ID NO:45)

```

5      3701  CTCGTTTTTT CTGTTGGTAC AAACCTTAATT GATTTTGAAT AATTTGTTTG
      3751  TACCAGTCCT TTTTGCTTAG CCCAGTCAAA ATAACGTTTG ATTGAATTAA
      3801  TGCGCCGGTT AATCGTAGAA GGTTTTAGTA ATCTTGTAAC TTGCATATGC
10     3851  CCTCGATATC GAGCAATAGT GCGAGCGGTA ACTTCTATTG GATGAAAAAG
      3901  AGTATCCTCA GCATGTTTTTC CCCACACATT TTCAAACCAA AATACAAAAT
      3951  CTTTTAAATC ACTCGTATAT TCTTTTAGTG TTTTGTATG CAAATCTCCT
15     4001  TCTTGAGATA AGCTAGAAAT AAAATCGGAA ATCAAAGATG TTGCTTGTAT

      4051  AGAAATTGTT TTAGTGGAAT GCATAAATAC CTCCTCTTTT ATTGACTTAC
                                     -35
20     4101  ATTAGCGGAC ATGATATTTT AATCTTATCA ATTATGTTAG CGGACATCAA
                                     -10      *→ relBEBt mRNA
      4151  ACATTTATTT TCCCACACTT CATGTCCACT AATATTAATT AGTGGACATT
                                     ----- --→ IR ←-----
                                     SD      Start relB
      4201  TAAAACTATC TCGAAAGTAG GTGTAACACA TGGCTATTCTG TAAAGATGAA
30     4251  TTGTATCGGT TAATTGATCA CCTGGATCAA CAAGATGAAA AAGCAGCATT

      4301  TGACTTTTTA GAATTTCTTG TTCAACGGTC AAGAAGAAAA CCTAAAGAAT
35     4351  GGGAAAAAAT TGATATGGCA GATCCTGATC ATGAACCGCT GTCTACACAA

      4401  GAGTTAGAAC AGTTAAACAG TGAAGAAGGA TATGTATCAG GGGAGGACGC
40     4451  AAAACGTGAA TTCGGACTAC AAATTGATTT ACCATAAGTC CGCGGTGAAA
                                     End relB      Start relE
45     4501  TTTATTGCAA AGCAAGAAAA AGGGATTCAA AAAAGAATTG CAGAAGGATT

      4551  GAAGGGACTT CTTAAGATTC CTCCTGAAGG AGATATTAAA AGTATGAAAG
50     4601  GTTACACAGA ACTATATCGA TTACGGATTG GAACCTTTCTG AATTTTATTT

```

4651 GAAATAAATC ATGATGAGAA AGTCATATAC ATACAAGCAA TTGGAAATCG

5                                      End *relE*

4701 TGGTGACATC TATAAA**TAAG** GCAAACATGC ATTTTAAAA GAAAGGTCTT

4751 CTGAATCGAA GAACCTTCCT TTTTGTGTG CGAATAATGT CCGCTAATGC

10    4801 TTGTTGCGTG ATTCTGTTCC ATTGCTACAC ATACCCC  
         (database accession number U03554)



#### 1.4. The archaeon *Methanococcus jannaschii* encodes a *relBE* homologous system

Again using database searching it was found that the completely sequenced genome of the methanogenic archaeon *Methanococcus jannaschii* codes for three *relE* homologous genes, one of which are located just downstream of *relB* homologous genes. This finding was surprising since, in many respects, archaeal organisms are more similar to eukaryotes than to bacteria (e.g. in their macromolecular synthesis apparatuses).

10 The DNA sequence of the *relBE*<sub>Mj1</sub> system is shown in Table 1.8. In this Table start codons and stop codons are shown in bold. The DNA sequence is from Bult et al., 1996.

Gene *relE*<sub>Mj1</sub> codes for a very basic polypeptide of 90 aa (pI = 11.0) and gene *relB*<sub>Mj1</sub> codes for an acidic polypeptide of 82 aa (pI = 4.4). The aa sequences of the RelE<sub>Mj1</sub> and RelB<sub>Mj1</sub> proteins are aligned with the other RelBE homologues in Tables 1.5 and 1.6, respectively. Thus, these basic similarities suggested that the *relBE*<sub>Mj1</sub> system may carry out similar or related functions in bacteria and archae. The properties of the second and third *relE* homologues of *M. jannaschii* are also given in Table 1.3. These comparisons show that *M. jannaschii* codes for one complete *relBE* homologous gene system and for two *relE* homologues without an adjacent *relB* partner.

Table 1.8. DNA sequence of a *relBE* homologous gene system from the archaeon *Methanococcus jannaschii* (SEQ ID NO:46)

5	751	CCGATACCGT	TGCTGGAGAC	ATAGCTGGAG	CTTTGAAGGC	GGAGAAGCTT
	801	ATTTTAATAA	CAGATGTTGA	TGGAATAATG	GATGATATAA	ATAATCCAGA
	851	GACGTTGCAT	AGAAAATTAA	CAGCTTCAGA	ACTAAAAGAA	ATGATAGAAG
10	901	ATGGAAGAAT	AAAGGGAGGG	ATGATTCCAA	AGGCTGAAAG	TGCCTTATAT
	951	GCCTTAGAGC	ATGGAGTTAA	GAGCGTTCAT	ATAATAAATG	GAAAGATTCC
	1001	TCATGCTTTG	TTGTTGGAGA	TATTTACAGA	GGAGGGTATT	GGGACGATGA
15	1051	TAACAAGAGA	TTAAAGTTTT	TATATTATAA	ACTACTTAAG	AATTAAAATA
				Start <i>relB<sub>Mj1</sub></i>		
	1101	AGACAAATAA	GGGGATAACT	<b>ATGCT</b> CAATA	TAAACAAAGA	GATAGCACAA
20	1151	ATAGAAACTG	AATTGAATGA	ATTGAAAAAA	TTGAGAGATG	AAATCTCTGA
	1201	AAGGATTGAA	AAATTAGAAA	TAAAGTTATT	AAAATTGAAA	GCATTAGCTA
	1251	TTCCAGAGGA	GGAATTTGAA	GAGGATTATG	AAGAAATTAT	AGAAGATGTT
25	1301	AAAAAATCTC	TGGATAAAAA	AGAGACTGTG	CCAGCAGAAG	AGGCTTTGAA
			End <i>relB<sub>Mj1</sub></i> /start <i>relE<sub>Mj1</sub></i>			
	1351	AGAATTGGGA	TTATT <b>ATGAA</b>	GTTTAACGTT	GAGATACATA	AAAGAGTCTT
30	1401	AAAAGATTTA	AAGGATTTGC	CTCCCTCAAA	CTTAAAGAAG	TTTAAAGAAC
	1451	TAATAGAAAC	ATTAAAAACC	AATCCCATTC	CAAAGAAAAA	ATTTGATATT
	1501	AAAAGATTAA	AAGGCAGTGA	TGAGGTTTAT	AGAGTTAGAA	TTGGAAAATT
35	1551	TAGAGTTCAA	TATGTTGTTT	TATGGGATGA	TAGAATAATA	ATAATTAGAA
				End <i>relE<sub>Mj1</sub></i>		
	1601	AGATAAGTAG	AAGAGAAGGA	GCTTATAAAA	ATCCC <b>TAA</b> GC	TATTAAAAAT
40	1651	TCTAATGGCT	ACATTTTTAT	ATCTCTTTTC	TTAATTCAAA	TAGAAAAAAC
	1701	AGATTGGGCT	GATACCATGA	TTATTCTTTT	AGATTTAAAT	GGAACAATAG
			(database accession number U67464)			

### 1.5. *relBE* homologous genes are ubiquitous in prokaryotes

Further *relBE* homologous two-component systems were discovered. The corresponding RelB and RelE homologous proteins are aligned in Tables 1.5 and 1.6, respectively.

- 5 It appears that *relE* homologous genes are present in a wide variety of Gram-negative bacteria (*E. coli*, *H. influenzae*, *V. cholera*, *H. pylori* and *Synechosystis*), in Gram-positive bacteria (*B. thuringiensis* and *M. tuberculosis*) and in *Archae* (*M. jannaschii* and *A. fulgidus*). Most strikingly, the archaeon *A. fulgidus* contains four complete *relBE* homologous gene systems.

10

- A number of features become evident from the alignments of the proteins (Tables 1.5 and 1.6) and from the properties listed in Tables 1.3 and 1.4. First, all RelE homologues are basic with pH's around 8-10 whereas the *RelB* homologues are acidic with pI's about 4-5. Secondly, the RelE proteins are in general slightly larger (90-120 aa)  
 15 than the *RelB* homologues (70 - 80 aa). Thirdly, the start codons of the *relE* genes are juxtaposed or even overlap with the stop codons of the linked *relB* partner, thus indicating translational coupling of *relE* to *relB*. These properties suggest that the proteins could exert similar functions in very different organisms.

## 20 EXAMPLE 2

### **Demonstration of translation of the *relB*<sub>K-12</sub> and *relE*<sub>K-12</sub> genes**

- Using the low copy-number *lacZ* fusion vector pOU253 (Table 0.1) in frame gene fusions between *relB*<sub>K-12</sub> and *relE*<sub>K-12</sub> and the *lacZ* gene were constructed (see Materials  
 25 and methods). Thus plasmid pKG4001 (+ 388 to + 596) carries a fusion between *relB*<sub>K-12</sub> and *lacZ*, and pKG4002 (+ 388 to + 921) carries a fusion between *relE*<sub>K-12</sub> and *lacZ*. The structure of the relevant parts of these reporter plasmids are shown in Fig. 1. When present in strain MC1000, both plasmids expressed significant amounts of  
 30 galactosidase fusion proteins, indicating that genes *relB*<sub>K-12</sub> and *relE*<sub>K-12</sub> are translated (Fig. 1). The *relE*<sub>K-12</sub>-*lacZ* fusion (pKG4002) expressed significantly lower amounts of  $\beta$ -galactosidase than the *relB*<sub>K-12</sub>-*lacZ* fusion, mainly because pKG4002 encodes an intact *relB*<sub>K-12</sub> gene which produces the RelB<sub>K-12</sub> autorepressor which inhibits transcription from the *relB* promoter.

## EXAMPLE 3

**Demonstration of translation of the *relB*<sub>P307</sub> and *relE*<sub>P307</sub> genes**

- 5 To detect authentic RelB<sub>P307</sub> and RelE<sub>P307</sub> proteins, in vitro translation reactions were carried out using high copy number pUC-plasmids carrying genes *relE*<sub>P307</sub> (pHA403), *relB*<sub>P307</sub> (pHA402) or both genes (pHA100) (for construction of these plasmids, see Materials and Methods and Table 0.1). Proteins produced in the *in vitro* translation reactions were labelled with <sup>35</sup>S-Methionine and separated by SDS-page. Fig. 2 shows
- 10 the direct visualization of RelB<sub>P307</sub> and RelE<sub>P307</sub>, thus providing evidence that the corresponding genes are translated.

## EXAMPLE 4

**15 Demonstrating that *relE*<sub>K-12</sub> is a cytotoxin**

- The low copy-number cloning vector pNDM220 contains *lacIq* and the LacI regulated pA1/O4/O3 promoter (Lanzer and Bujard, 1988) upstream of a multiple cloning site (mcs). The genetic structure of pNDM220 is shown in Fig. 3. Without IPTG added to
- 20 the growth medium, the pA1/O4/O3 promoter is almost completely turned off. However, with IPTG, strong transcription is induced towards the cloning site. Therefore plasmid pNDM220 is suitable for the conditional expression of genes, in particular toxin-encoding genes.
- 25 The *relE*<sub>K-12</sub> gene of *E. coli* K-12 (Bech et al., 1985) was PCR amplified and inserted into the mcs of pNDM220, resulting in pMG223 (for the construction of pMG223, see Materials and methods). Plasmid pMG223 (+ 733 to + 1020) was established in MC1000, which contains a chromosomal copy of the *relBE* operon. However, it was not possible to transform pMG223 into the JS115 strain, which carries a deletion of
- 30 the *relBE* operon ( $\Delta relB$ ). Therefore, the induction experiments shown in Fig. 4 were accomplished using strain MC1000, which contains the chromosomal copy of *relBE*<sub>K-12</sub>.

Strain MC1000/pMG223 was grown in LB at 37°C. At time zero, IPTG was added to the growth-medium. After two hours of induction with IPTG, the viable counts decreased c. 600-fold (Fig. 4B). The decline started immediately and continued exponentially for about 2 hours. On plates containing IPTG, viable counts decreased even further (data not shown). The optical density (OD<sub>450</sub>) increased during the first 20 minutes after addition of IPTG and then the culture became stationary (Fig. 4A). Addition of IPTG to growing cells containing the vector-plasmid had no effect (not shown). These results indicate that the *relE* gene encodes a cell toxin.

## 10 EXAMPLE 5

### Demonstrating that RelB<sub>K-12</sub> is an antitoxin

Plasmid pMG2202 (+ 388 to + 921) is a pBR322 derivative that contains the *relB* gene expressed from its own promoter (see Table 1.1). Plasmid pMG2201 (+ 388 to + 597) is a pBR322 derivative that contains the *relB* promoter and the first part of *relB*<sub>K-12</sub>. Thus, pMG2201 does not contain an intact *relB* gene and was included in the analyses as a control plasmid. The strains MC1000/pMG223 (pA1/O4/O3::*relE*+) /pMG2202 (*relB*<sup>+</sup>) and MC1000/pMG223/pMG2201 (*relB*) were subjected to a physiological growth experiment similar to the one described in Example 4. As seen from Fig. 4E and 4F, the presence of the high copy-number *relB*-carrying plasmid suppressed *relE*- dependent cell killing. The antitoxin effect was dependent on an intact *relB* reading frame, since the control-plasmid (pMG2201) carrying the promoter region and the first part of the *relB* reading frame did not prevent the *relE* mediated cell killing (Fig. 4C, 4D).

## EXAMPLE 6

### Demonstrating that *relE*<sub>P307</sub> encodes a very efficient cytotoxin

30

The medium copy number expression vector pBAD33 contains an arabinose inducible promoter (pBAD) with a multiple cloning site (mcs) and the *araC* gene (Guzman et al., 1995). The genetic structure of pBAD33 is shown in Fig. 5. Without arabinose added to the growth medium, the pBAD promoter is completely turned off. However, with

arabinose, strong transcription is induced towards the cloning site. On top of this property, the pBAD promoter is repressible by the addition of glucose to the growth medium. Thus, by the addition of glucose, transcription from pBAD can be rapidly and efficiently turned off.

5

The glucose repression effect is epistatic to the inducer effect by arabinose. Hence, if cells with a pBAD-carrying plasmid are grown in a medium containing both arabinose and glucose then the promoter is not induced. However, if cell-growth depletes the medium for glucose, then the promoter will be induced. Therefore, plasmid pBAD33 is  
10 suitable for the conditional turning on and off of the expression of genes, in particular toxin-encoding genes as described herein.

The *relE* gene of the *E. coli* plasmid P307 (Saul et al., 1989) was PCR amplified and inserted into the mcs of pBAD33, resulting in pHA810 (for the construction of plasmid  
15 pHA810, see Materials and methods). Thus plasmid pHA810 contains the *relE*<sub>P307</sub> gene inserted downstream of the pBAD promoter. Strain MC1000/pHA810 was grown in LB-medium without glucose at 37°C. At time zero, the culture was diluted into medium containing either 0 or 0.2% arabinose. In the arabinose-containing culture, an immediate decline in viable counts was observed (Fig. 6B, closed symbols).  
20 The decline continued exponentially throughout the experiment. After 240 min of induction with arabinose, viable counts had decreased more than five orders of magnitude. Without arabinose, cells containing pHA810 continued to grow exponentially (Fig. 6A and 6B, open symbols). On plates containing arabinose, none or very few viable cells were detected. These results show that *relE*<sub>P307</sub> gene encodes an extremely  
25 efficient cell toxin.

## EXAMPLE 7

### Demonstrating that *RelB*<sub>P307</sub> is an antitoxin

30

Plasmid pHA110 (+ 1 to + 1122) is a pBR322 derivative that contains the *relB*<sub>P307</sub> gene expressed from its own promoter. The strain MC1000/pHA810/pHA110 (*relB*<sub>P307</sub>) was subjected to a physiological growth experiment as described in Example 6. It appeared that the presence of the *relB*<sub>P307</sub>-carrying plasmid pHA110 prevented

*relE*<sub>P307</sub> dependent inhibition of cell growth (Fig. 6C) and cell killing (Fig. 6D). This observation shows that *relB*<sub>P307</sub> codes for an antitoxin that counteracts the cell killing caused by RelE<sub>P307</sub>.

## 5 EXAMPLE 8

### Determination of the frequency of spontaneous mutants that are resistant to the killing effect of RelE

10 Strain MC1000/pHA810 was grown exponentially to an OD of 0.5 and serial dilutions of the cell suspension were plated on LA plates containing chloramphenicol (selecting for plasmid pHA810) and with or without 0.02% arabinose (which induces expression of *relE* present in pAH810). On such plates without arabinose the plating efficiency of strain MC1000/pAH810 was normal, i.e. more than 99% of the viable cells produced  
15 a colony. This indicated that the presence of pAH810 in itself had no effect on the viability of the cells. However, with arabinose the plating efficiency was reduced by about 10<sup>9</sup> fold, thus indicating that expression of RelE is extremely toxic to the cells. The few surviving colonies that appeared eventually were retransformed with the RelE expression plasmid pHA210 which can co-exist with pAH810. However, none of the  
20 surviving cells from the first round of selection (i.e. using pHA810) survived induction of RelE (by addition of IPTG) from the second plasmid pAH210.

These results show that resistance against RelE toxicity is a very rare event, as based on this experiment it is less than about 10<sup>-9</sup>.

25

## EXAMPLE 9

### Demonstrating that RelE of the Archeon *Methanoccus jannaschii* is toxic to *E. coli*.

30 The *relE* gene of *M. jannaschii* was amplified from genomic DNA using primers MJ-relE/2CWW (5'-CCCCCGAATTTCGCATGCGCCATTAGAAT, SEQ ID NO:47) and MJ-relE/1CW (5'-CCCCCGGATCCGAGCTCGAGGCTTTGAAAGAATTGGG, SEQ ID NO:48). The resulting DNA fragment was cleaved with *Bam*HI and *Eco*RI and cloned into plasmid pNDM220 (Fig. 3) thus yielding pHA705 (Fig. 7). Similarly, *relB* and *relE*

from *M. jannashii* were PCR amplified using primers relB-M.jannCW (5'-CCCCGGATC-CGTGACGACAAATAAGGGGATAACTATG, SEQ ID NO:49) and MJ-reIE/2CWW. The resulting DNA fragment was cleaved with *Bam*HI and *Eco*RI and cloned into pNDM220, thus yielding pHA715 (Fig. 7).

5

Plasmids pHA705 (carrying *relE*) and pHA715 (carrying *relBE*) were transformed into *E. coli* K-12 strain MC1000. Cells were grown exponentially and followed after the addition of IPTG. Fig. 8 shows that the addition of IPTG inhibited the growth of MC1000/pHA705 but not that of MC1000/pHA715, and Fig. 9 shows that viable  
10 count was significantly reduced in the case of MC1000/pHA705 but not in that of MC1000/pHA715, thus demonstrating that RelE of *M. jannashii* is toxic to *E. coli*.

#### EXAMPLE 10

#### 15 Demonstrating that RelE of the Gram-positive bacterium *Streptococcus pneumoniae* is toxic to *E. coli*.

Using BLAST database searching, we identified two homologues of the *relBE* genes of *S. pneumoniae*. The DNA sequence of the homologue designated *relE*<sub>Sp2</sub> is shown in  
20 Fig. 10. Gene *relE*<sub>Sp2</sub> was PCR amplified from genomic DNA of *S. pneumoniae* strain RP46 using primers relE-Sp2/cw (5'-CCCCGGATCCGATGCATGATTTAGGCTTGAAG, SEQ ID NO:50) and relE-Sp2/ccw (5'-CCCCGAATTCGAATGAAA-ATTTACTTGAAAAAAG, SEQ ID NO:51). The resulting DNA fragment was cleaved with *Bam*HI and *Eco*RI and cloned into pNDM220 thus yielding plasmid pHA-Sp2 (Fig.  
25 11).

Plasmid pHA-Sp2 (carrying *relE*<sub>Sp2</sub>) was transformed into *E. coli* strain MC1000. Cells were grown exponentially and followed after the addition of IPTG. Fig. 8 shows that the addition of IPTG inhibited the growth of MC1000/pHA-Sp2, and Fig. 9 shows that  
30 viable counts were dramatically reduced, thus demonstrating that expression of *relE*<sub>Sp2</sub> is highly toxic to *E. coli*.



## EXAMPLE 11

**Cloning of the *relE* genes of plasmid P307, *M. jannashii* and *E. coli* K-12 into the broad-host-range vector pHAG33.**

5

The broad-host-range vector pVLT33 is an RSF1010 derivative that can be mobilized by an appropriate conjugation system (de Lorenzo, Eltis, L., Kessler, B. and Timmis, K.N. 1993. Analysis of *Pseudomonas* gene products using *lac<sup>R</sup>/Ptrp-lac* plasmids and transposons that confer conditional phenotypes. Gene 123, 17-24). It also contains the *tac*-promoter (*ptac*) and *lac<sup>R</sup>*. Since *ptac* is leaky and therefore unsuitable for the regulated expression of toxins, the promoter was replaced by the pA1/O4-O3 promoter of pNDM220. The resulting plasmid, pHAG33, is shown in Fig. 12. The *relE* genes of pHA210 (*relE<sub>P307</sub>*), pHA705 (*relE<sub>Mj</sub>*) and pMG223 (*relE<sub>K12</sub>*) were cloned into pHAG33, resulting in plasmids pHAG33-2 (Fig. 13), pHAG33-3 (Fig. 14), and pHAG33-4 (Fig. 15), respectively.

## EXAMPLE 12

**Demonstrating that RelEs of *E. coli* K-12, P307 and *M. jannashii* are toxic to *Pseudomonas putida*.**

20

Plasmids pHAG33-2 (*relE<sub>P307</sub>*), pHAG33-3 (*relE<sub>Mj</sub>*) and pHAG33-4 (*relE<sub>K12</sub>*) were transformed into the *E. coli* K-12 strain S17-1. This strain contains the conjugation system of RP4 and is thus able to mobilize pHAG33-derived plasmids as described above (Simon et al., 1986). After conjugation on solid medium to *P. putida* strain KT2440 according to standard procedure, strains KT2440/pHAG33-2, KT2440/pHAG33-3 and KT2440/pHAG33-4 were established.

The strains were grown exponentially in LB containing 30 µg/ml ampicillin and 50 µg/ml kanamycin and followed after the addition of 2 mM IPTG. As seen from Fig. 16, the increment in cell-growth as measured by OD<sub>450</sub> was reduced by IPTG in all three cases. Furthermore, measurements of viable counts (Fig. 17) showed cell-killing in all three cases, most severe in the case of *relE<sub>K12</sub>* (pHAG33-4(+)) in Fig. 17). Thus, RelE

30

proteins of P307 and *M. jannashii* are toxic to *P. putida* and RelE of *E. coli* K-12 is extremely toxic to *P. putida*.

#### EXAMPLE 13

5

##### **Demonstrating biological containment by the depletion of a carbon source.**

Plasmid pHA810 (Fig. 18) was constructed by inserting the *relE* gene of P307 into the expression vector pBAD33 (Fig. 5). The promoter (designated p<sub>BAD</sub>) upstream of  
10 *relE*<sub>P307</sub> in pHA810 is repressed by glucose and induced by arabinose. The repression by glucose overrides induction by arabinose such that the simultaneous presence of glucose and arabinose in the growth medium results in repression of the promoter.

To simulate a realistic scenario in which the carbon source was depleted, we grew  
15 MC1000/pHA810 in ABT minimal salts medium at 35°C in the presence of a limiting amount of glucose (0.025% w/v) (represses p<sub>BAD</sub>) and varying the concentration of arabinose (induces p<sub>BAD</sub>). Optical density (Fig. 19) and viable counts (Fig. 20) typical for such an experiment were obtained. As seen in Fig. 19, the rate of increase in OD<sub>450</sub> is severely reduced by the highest amounts of arabinose (0.050% and  
20 0.075%). This was expected, since arabinose induces pBAD and the limited amount of glucose (0.025%) cannot fully suppress pBAD at high concentrations of arabinose. The glucose added (0.025%) was depleted by cell growth at an OD<sub>450</sub> = approx. 0.1. At this OD<sub>450</sub>, a dramatic cell killing was seen in the case of 0.005%, 0.010%, and 0.025% of arabinose (Fig. 20). This result shows, that depletion of the carbon source  
25 (glucose) leads to massive cell killing, and thus to biological containment of the plasmid that carries *relE*<sub>P307</sub>.

#### EXAMPLE 14

##### **30 Demonstrating that RelE of *E. coli* K-12 and *M. jannashii* are toxic to human cells.**

The cell line 293 is a permanent line of primary human embryonal kidney cells transformed by human adenovirus type 5 (Ad 5) DNA (ATCC CRL-1573). The cells are particularly sensitive to human adenovirus, are highly permissive for adenovirus DNA, and

contain and express the transforming genes of AD 5 (Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59-74).

- 5 Plasmid pcDNA3.1(+) (Invitrogen) carries the constitutive promoter  $P_{CMV}$  from cytomegalovirus upstream of a multiple cloning site (mcs). Genes *relE* of *E. coli* K-12 and *M. jannashii* were inserted in the mcs, resulting in plasmids p5.4 and p5.3, respectively.
- 10 Plasmids pcDNA3.1(+)(control), p5.4 and p5.3 were transfected into cell line 293 by selection in medium containing G418 (geneticin), which selects for cells expressing the neomycin gene present on the plasmids. After 12 days, the cell density was measured by inspection. In the case of p5.4 (*relE*<sub>K-12</sub>), between 0 and 5% of the cells had survived (as compared to the control). In the case of p5.3 (*relE*<sub>Mj</sub>), between 5 and
- 15 10% of the cells had survived. These results indicate that the bacterial RelE<sub>K-12</sub> and the archaeal RelE<sub>Mj</sub> toxins both are lethal to human cells.

#### EXAMPLE 15

#### 20 Demonstrating that RelE<sub>K-12</sub>, RelE<sub>P307</sub>, and RelE<sub>Mj</sub> inhibit translation in vitro

DNA fragments comprising genes *relE*<sub>K-12</sub>, *relE*<sub>P307</sub> and *relE*<sub>Mj</sub> were PCR amplified such that a T7 RNA polymerase promoter was placed upstream of the corresponding genes (according to Thisted et al., 1994).

25

The following primers were used: *relE*<sub>K-12</sub> (P1: 5'- TGTAATACGACTCACTATAGATAAGGAGTTTTATAAATGGCGTATTTCTGGATTTG, SEQ ID NO:52) and P2 (CACCTTCGGTGCGAAACAG, SEQ ID NO:53); *relE*<sub>P307</sub> (P3: 5'-TGTAATACGACTCAC-TATAGATAAGGAGTTTTATAAATGAGGTATCAGGTAAAATTCA (SEQ ID NO:54) and

- 30 P4: 5'-CTTTCCATCGGCGAATTATC, SEQ ID NO:55); *relE*<sub>Mj</sub> (P5: 5'-TGTA-ATACGACTCACTATAGATAAGGAGTTTTATAAATGAAGTTTAACGTTGAGATAC SEQ ID NO:56) and P6: (5'-ATCATGGTATCAGCCGAATC, SEQ ID NO:57). T7 RNA polymerase sequences are underlined, and the strong Shine-Dalgarno (SD) sequence from the *parA* system of plasmid R1 is shown in italics.

Using in vitro transcription with T7 RNA polymerase according to standard procedures, mRNAs encoding *relE*<sub>K-12</sub>, *relE*<sub>P307</sub>, and *relE*<sub>Mj</sub> were produced and subsequently purified from a denaturing polyacrylamide gel. To facilitate the quantification of the mRNAs they were labelled with tritium (alfa-<sup>3</sup>H-CTP) during their synthesis. The *relE*-  
5 encoding mRNAs (1.5 pmol) were used as templates in in vitro translation reactions employing an S30 extract (obtained from Promega) containing 150 µM of each amino acid except Methionine which was 1 µM.

Figure 21 shows SDS-PAGE (tricine-gel) analysis of such an experiment. The in vitro  
10 translation reactions were initiated with unlabelled Methionine in order to produce RelE toxin in the reaction. Ten minutes after the addition of the unlabelled Methionine, radioactive 35-S-Methionine (5 pmol in a 15 µl volume) was added and the reaction continued for an additional 20 minutes. C in Fig. 21 denotes a control lane without exogenous mRNA added. The protein bands seen in this lane originate from translation  
15 of mRNAs present in the S30 extract. In lane 1, the *in vitro* translation reaction contained an mRNA encoding the *relE* gene of *E. coli* K-12. As seen, the translation reaction was severely inhibited. In lane 2, a mRNA encoding a mutated *relE* (denoted *relEmE* and described in Gotfredsen et al, 1998) gene was added. As seen, the presence of this mRNA did not inhibit the reaction. This result shows that the RelE protein  
20 produced during the initial incubation-period without 35-S-Met added inhibits the in vitro translation reaction (i.e. compare lanes 1 & 2). Furthermore, this lack of inhibition is correlated with loss of cell killing activity in vivo (since the mutated *relE* gene, *relEmE*, used in lane 2 is not toxic to *E. coli* cells), thus indicating that inhibition of translation is the actual cause of cell death in vivo. In lanes 3 and 4, mRNAs encoding  
25 *relE* of plasmid P307 and the archaeon *M. jannashii* were added. As seen, the presence of these mRNAs inhibited the in vitro translation reactions as well. These results indicate that the RelE toxins from *E. coli* K-12, *M. jannashii* and plasmid P307 all act by inhibition of translation.

## EXAMPLE 16

**Demonstrating that RelE<sub>K-12</sub> is toxic to yeast cells.**5 1. Yeast strain

In these experiments the yeast strain *Saccharomyces cerevisiae* 281288DIV-36 (MATa his 4-5; LEU2 THR4 ura3-52 trp1 CYH2 KAR1) was used.

10 2. PCR amplification of RelE coding region

The RelE coding region was PCR amplified from the plasmid pMG223 using two oligonucleotide primers. The primer S-RelE was 24 nucleotides long (5'-TAGGTACCATGGCGTATTTTCTGG-3', SEQ ID NO:58). It contains *KpnI* and *NcoI* endonuclease restriction sites at the 5' end with an 8 nucleotide overhang. Primer AS-RelE was 23 nucleotides long (5'-GAGACCCACACTACCATCGGCG-3', SEQ ID NO:59) and hybridises 400 nucleotides downstream the RelE termination codon and 392 nucleotides downstream the *EcoRI* site in plasmid vector pMG223. PCR amplifications were performed using Vent® Polymerase (New England Biolabs), 200 μM of each dNTP. PCR reaction buffer (10 mM KCl; 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 20 mM Tris-HCl (pH 8.8); 2 mM MgSO<sub>4</sub>; 0.1% Triton X-100) with 0.2 μM of each of the primers. After 5 min denaturation (95°C) PCR was performed with 20 cycles, each cycle consisting of 1 min denaturation (92°C), 1 min primer annealing (50°C) and 1 min primer extension (72°C). Successful PCR products of 706 bp DNA fragments were identified and purified from a 1% agarose gel after a run of 1 hour at 40 mA. The PCR product was digested with the two restriction enzymes *KpnI* and *EcoRI* and the fragment of 304 bp containing the RelE open reading frame was isolated after electrophoresis on a 1.2 % agarose gel, and purified using a gel extraction kit (Pharmacia).

### 3. Cloning of the amplified *relE* gene

The isolated DNA fragment of the *relE* gene flanked with *KpnI* and *EcoRI* sites was ligated into the pYES2 expression vector (Invitrogen) previously digested with *KpnI* and *EcoRI* using standard procedures (Sambrook). After ligation, *E. coli* Top10 (Invitrogen) was transformed with the ligation mixture using electroporation using the *E. coli* gene pulser (BioRad). After phenotypic expression for 2 hours in SOC medium the culture was spread onto selective LB medium (Sambrook) containing 50 µg of ampicillin per ml. Transformed colonies were identified using PCR amplification and a positive clone designated pPK727 was further tested by restriction enzyme analysis. The functionality of the PCR amplified *RelE* gene was tested in *E. coli* by cloning the *NcoI-EcoRI* fragment from pPK727 into the *E. coli* expression vector pUHE24. Induction with IPTG led to cell killing in *E. coli*.

### 4. Yeast transformation

*S. cerevisiae* was grown overnight (ON) in YDP medium (1% yeast extract; 2% Bacto peptone; 2% glucose). For a single transformation, cells from 1 ml ON culture were spun down (5,000 rpm for 30 sec using an Eppendorf minicentrifuge) and washed twice in sterile water. Cells were resuspended in 200 µl lithium acetate buffer (10 mM Tris-HCl pH 7.6 with 100 mM LiOAc, 1 mM EDTA). After incubation for 15 min. at 25°C with agitation two transformations were made adding 20 µl carrier DNA (10 mg/ml salmon sperm DNA, sonicated and heat denatured) and 100 ng of the plasmids pPK727 and pYES2 (vector control), respectively. A volume of 1.2 ml 40% PEG 4,000 in 0.1 M lithium acetate buffer was added to each transformation mixture. The transformation mixtures were incubated in a 25°C incubator for 30 min before transferring to a 42°C water bath for 15 min. Cells were spun down (5,000 rpm for 30 sec.) and washed once with sterile water before plating on Uracil drop-out medium (1% Bernstein acid; 0.1% NaOH; 2% glucose; 0.67% Bacto yeast nitrogen base; 0.1% amino acids (without uracil); 2% agar).

After three days growth at 30°C single colonies were picked and streaked onto plates with Uracil drop-out medium. After two days at 30°C cells were transferred to induc-

tion medium (Uracil drop-out medium with 2% galactose as the sole carbon source) by replica-plating.

Single colonies of *S. cerevisiae* containing pPK727 and pYES2 were transferred to liquid Uracil drop-out medium (1% Bernstein acid; 0.1% NaOH; 2% glucose; 0.67% Bacto-yeast nitrogen base; 0.1% amino acids (without uracil)) and incubated ON. To compensate for difference in cell density, a volume of 50  $\mu$ l per OD<sub>540</sub> (optical density at 540 nm) was used to inoculate 50 ml of liquid Uracil drop-out medium with either glucose or galactose as sole carbon source, respectively. The four flasks with *S. cerevisiae* (pPK727) and *S. cerevisiae* (pYES2) in Uracil drop-out medium with or without galactose were incubated at 30°C with moderate shaking (200 rpm). To monitor growth, samples were taken at different time point and OD<sub>540</sub> was determined. Samples were taken in duplicates and the average OD<sub>540</sub> calculated and plotted against time of sampling

15

### 5. Results.

All colonies containing either the plasmid pPK727 or the pYES2 control plasmid were able to grow on plates with glucose as carbon source. When transferred to plates with galactose as the sole carbon source leading to gene expression from the P-gal1 promoter only cells with the pYES2 control plasmid showed normal growth, whereas cells containing the pPK727 were strongly inhibited in growth.

In liquid media, the yeast cells in which the *relE* gene was induced showed a remarked growth inhibition when compared to the uninduced control and to the controls with only the plasmid pYES2 without insert.

These results that are summarised in the below Table 16.1 clearly suggest that inhibition of cell growth in yeast cells is due to expression of the *relE* gene.

30

Table 16.1. Growth of *Saccharomyces cerevisiae* transformed with pYES2 + /- *relE* gene

time (hours)	Plasmid + relE		plasmid (control)	
	Galactose	no galactose	galactose	no galactose
0	0	0	0	0
5,5	0,018	0,024	0,023	0,028
15,5	0,013	0,186	0,055	0,176
23	0,190	1,195	0,286	0,963
28	0,010	1,480	0,816	1,649
29	0,035	3,930	1,660	2,650
65	0,990	3,950	6,180	3,820



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## CLAIMS

1. A method of conditionally controlling the survivability of a recombinant microbial cell population, the method comprising (i) providing in the cells of said population a  
5 gene coding for a cytotoxic first kind of polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally equivalent polypeptide (said genes collectively being designated herein as the *relE* gene family), said gene is expressible in the cells of the population and, operably linked to the gene, a regulatable regulatory DNA sequence and (ii)  
10 cultivating the cell population under conditions where the *relE* gene or the gene coding for a functionally equivalent polypeptide is expressed, the expression leading to an at least partial killing of the cell population.
2. A method according to claim 1 wherein the gene coding for the functionally  
15 equivalent polypeptide is derived from a Gram-negative bacterium.
3. A method according to claim 2 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp.,  
20 *Pseudomonadaceae* spp., *Helicobacter* spp. and *Synechosystis* spp.
4. A method according to claim 1 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium.
- 25 5. A method according to claim 4 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.
6. A method according to claim 5 wherein the Gram-positive bacterium is *Bacillus*  
30 *thuringiensis*.
7. A method according to claim 1 wherein the gene coding for the functionally equivalent polypeptide is derived from a species belonging to *Arhae*.

8. A method according to claim 1 wherein the *relE* family gene is present on the chromosome of the cells.
9. A method according to claim 8 wherein the cells of the population do not contain a gene coding for a second type of polypeptide that is capable of counteracting the cell toxic effect of the RelE polypeptide or the functional equivalent hereof.
10. A method according to claim 1 wherein the gene coding for the RelE polypeptide or the functional equivalent hereof is present on an extrachromosomal replicon.
- 10 11. A method according to claim 1 wherein the regulatory DNA sequence regulates the expression of the gene coding for the RelE polypeptide or the functionally equivalent polypeptide at the transcriptional level by means of a promoter, the function of which is regulated by the presence or absence of a chemical compound in the cultivation medium.
- 15 12. A method according to claim 11 wherein the promoter is inducible by a chemical compound.
- 20 13. A method according to claim 11 wherein the promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound whereby, when the first kind of compound is depleted from the medium, the promoter is induced by the second kind of compound.
- 25 14. A method according to claim 1 wherein the cells of the population comprises a gene coding for a second kind of polypeptide that is capable of binding to the *relE* polypeptide or the functional equivalent, said binding resulting in that the toxic effect of the RelE polypeptide or the functional equivalent is at least partially counteracted.
- 30 15. A method according to claim 14 wherein the gene coding for the second kind of polypeptide is operably linked to a regulatable regulatory DNA sequence, permitting that the gene coding for the second kind of polypeptide is suppressed under conditions where the gene coding for the RelE polypeptide or the functional equivalent is expressed.

16. A method according to claim 14 wherein the second kind of polypeptide is the RelB polypeptide derived from *E. coli* K-12 or a functionally equivalent polypeptide.

17. A method according to claim 16 wherein the functionally equivalent second kind  
5 of polypeptide is derived from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae* spp.

18. A method according to claim 1 wherein the expression of the *relE* gene family gene is stochastically regulated.

10

19. A method according to claim 18 wherein the stochastical regulation is effected by operably linking the *relE* gene family gene to a regulatory sequence that comprises an invertible promoter.

15 20. A method according to claim 18 wherein the stochastical regulation is effected by flanking at least part of the regulatory sequence by repeat sequences whereby the at least part of the regulatory sequence is recombinationally excised.

21. A method according to claim 1 wherein the cells contain a gene coding for a gene  
20 product of interest.

22. A method according to claim 21 wherein the gene product of interest is an immunologically active gene product.

25 23. A method according to claim 21 wherein the gene product of interest is one that is effective in degradation of an environmental pollutant.

24. A method according to claim 21 wherein the gene product of interest is a pesticidally active product.

30

25. A method according to claim 24 wherein the gene coding for the pesticidally active gene product is derived from *Bacillus thuringiensis*.

26. A method of confining an extrachromosomal replicon to a microbial cell population, the method comprising the steps of

- (i) isolating a microbial cell naturally containing a gene belonging to the *relE* gene family coding for a first kind of polypeptide that, when it is expressed in the cell, acts as a toxin for the cell or, if the cell does not naturally contain a gene belonging to the *relE* gene family, introducing such a gene into the cell,
- (ii) introducing into the cell the extrachromosomal replicon to be confined, said replicon containing a gene coding for a second kind of polypeptide that, by binding to the first kind of polypeptide, acts as an antitoxin for said first kind of polypeptide,
- (iii) cultivating the cell under conditions where the genes coding for the first and the second kind of polypeptides are expressed, whereby a daughter cell that does not receive a copy of the extrachromosomal replicon is killed by the first kind of polypeptide being expressed in the absence of expression of the second kind of polypeptide.

27. A method according to claim 26 wherein the cell population consists of cells that comprises a gene coding for a gene product of interest.

20

28. A method according to claim 27 wherein the gene product of interest is selected from the group consisting of an enzyme, an immunologically active polypeptide, a pesticidally active gene product and a pharmaceutically active gene product.

25 29. A method according to claim 26 wherein the replicon is a plasmid occurring in the microbial cells at a copy number which is in the range of 1-30 including the range of 1-10 such as the range of 1-5.

30. A method according to claim 26 wherein the microbial cells belong to a Gram-negative bacterial species.

30

31. A method according to claim 30 wherein the bacterial species is selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp.

32. A method according to claim 26 wherein the microbial cells belong to a Gram-positive bacterial species.

33. A method of post-segregationally stabilizing a plasmid in a microbial host cell  
5 population, the method comprising the steps of

(i) recombinationally inserting into the plasmid (a) a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a functional equivalent thereof, said first kind of polypeptide having a toxin effect  
10 on the host cell and (b) a gene coding for a second kind of polypeptide that (1) is capable of acting as an antitoxin for first kind of polypeptide and (2) is capable of being degraded in the host cell at a higher rate than that at which the first kind of polypeptide is degraded,

15 (ii) cultivating the cell population under conditions where the genes coding for the first kind and second kind of polypeptides are expressed,

whereby a daughter cell that does not receive at least one copy of the plasmid is killed as a result of the faster degradation of the second kind of polypeptide.

20

34. A method according to claim 33 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium.

35. A method according to claim 34 wherein the gene coding for the functionally  
25 equivalent polypeptide is derived from a Gram-negative bacterium selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp., *Pseudomonadaceae* spp., *Helicobacter* spp. and *Synechosystis* spp.

36. A method according to claim 33 wherein the gene coding for the functionally  
30 equivalent polypeptide is derived from a Gram-positive bacterium.

37. A method according to claim 36 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.



38. A method according to claim 37 wherein the Gram-positive bacterium is *Bacillus thuringiensis*.

39. A method according to claim 33 wherein the gene coding for the functionally  
5 equivalent polypeptide is derived from a species belonging to *Arhae*.

40. A method according to claim 33 wherein the second kind of polypeptide is selected from the group consisting of the relB polypeptide derived from *E. coli* K-12 and a functionally equivalent polypeptide.

10

41. A method according to claim 40 wherein the functionally equivalent second kind of polypeptide is derived from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae* spp.

15 42. A method according to claim 33 wherein the plasmid contains a gene coding for a gene product of interest.

43. A method according to claim 42 wherein the gene product of interest is an immunologically active gene product.

20

44. A method according to claim 42 wherein the gene product of interest is one that is effective in degradation of an environmental pollutant.

45. A method according to claim 42 wherein the gene product of interest is a pesti-  
25 cally active product.

46. A method according to claim 45 wherein the gene coding for the pesticidally active gene product is derived from *Bacillus thuringiensis*.

30 47. A method according to claim 33 wherein the microbial cells belong to a Gram-negative bacterial species.

48. A method according to claim 47 wherein the bacterial species is selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp.
- 5 49. A method according to claim 33 wherein the microbial cells belong to a Gram-positive bacterial species.
50. A method according to claim 33 wherein the plasmid is one that occurs in the microbial cells at a copy number which is in the range of 1-30 including the range of 1-  
10 10 such as the range of 1-5.
51. A recombinant cell comprising a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally equivalent polypeptide or a variant or derivative hereof, said  
15 first kind of polypeptide having a toxic effect on the cell, subject to the limitation that when the cell is *E. coli*, the gene coding for the first kind of polypeptide is not derived from *E. coli*.
52. A cell according to claim 51 wherein the cells belong to a bacterial species selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vi-*  
20 *brionaceae* spp. and *Pseudomonadaceae* spp.
53. A cell according to claim 51 wherein the cells belong to a Gram-positive bacterial species.  
25
54. A cell according to claim 51 wherein the gene coding for *E. coli* K-12 RelE polypeptide or the functional equivalent hereof is located on the chromosome.
55. A cell according to claim 51 wherein the gene coding for the *E. coli* K-12 RelE  
30 polypeptide or the functional equivalent hereof is located on an extrachromosomal replicon.
56. A cell according to claim 51 which comprises at least one gene coding for a gene product of interest.

57. A cell according to claim 56 wherein the gene product of interest is an immunologically active gene product.

58. A cell according to claim 56 wherein the gene product of interest is one that is  
5 effective in degradation of an environmental pollutant.

59. A cell according to claim 51 wherein the gene product of interest is a pesticidally active product.

10 60. A cell according to claim 51 which comprises a gene coding for a second kind of polypeptide that is capable of binding to the first kind of polypeptide whereby the toxic effect hereof is at least reduced.

61. A cell according to claim 51 comprising, operably linked to the gene coding for a  
15 first kind of polypeptide, a regulatable regulatory DNA sequence.

62. A cell according to claim 61 wherein the regulatory DNA sequence regulates the expression of the gene coding for the first kind of polypeptide at the transcriptional level by means of a promoter, the function of which is regulated by the presence or  
20 absence of a chemical compound in the cultivation medium.

63. A cell according to claim 62 wherein the promoter is inducible by a chemical compound.

25 64. A cell according to claim 62 wherein the promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound whereby, when the first kind of compound is depleted from the medium, the promoter is induced by the second kind of compound.

30 65. A cell according to claim 51 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium.

66. A cell according to claim 65 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium selected from the group

consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp., *Pseudomonadaceae* spp., *Heliobacter* spp. and *Synechosystis* spp.

67. A cell according to claim 51 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium.

68. A cell according to claim 67 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.

10

69. A cell according to claim 68 wherein the Gram-positive bacterium is *Bacillus thuringiensis*.

70. A cell according to claim 51 wherein the gene coding for the functionally equivalent polypeptide is derived from a species belonging to *Arhae*.

15

71. A cell according to claim 51 which does not contain a gene coding for a second type of polypeptide that is capable of counteracting the cell toxic effect of the first kind of polypeptide.

20

72. A cell according to claim 51 which comprises a gene coding for a second kind of polypeptide that is capable of binding to the first kind of polypeptide, said binding resulting in that the toxic effect of the first kind of polypeptide is at least partially counteracted.

25

73. A cell according to claim 72 wherein the gene coding for the second kind of polypeptide is operably linked to a regulatable regulatory DNA sequence, permitting that the gene coding for said second kind of polypeptide is suppressed under conditions where the gene coding for the RelE polypeptide or the functional equivalent is expressed.

30

74. A cell according to claim 72 wherein the second kind of polypeptide is the RelB polypeptide derived from *E. coli* K-12 or a functionally equivalent polypeptide.

75. A cell according to claim 74 wherein the functionally equivalent second kind of polypeptide is derived from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae* spp.

5 76. A cell according to claim 61 wherein the expression of the gene coding for the first kind of polypeptide is stochastically regulated.

77. A cell according to claim 76 wherein the stochastic regulation is effected by operably linking the gene coding for the first kind of polypeptide to a regulatory sequence  
10 that comprises an invertible promoter.

78. A cell according to claim 76 wherein the stochastic regulation is effected by flanking at least part of the regulatory sequence by repeat sequences whereby the at least part of the regulatory sequence is recombinationally excised.

15

79. A composition comprising a cell according to any of claims 51-78.

80. A method of limiting the survival of a cell population in a first or a second environment, which method comprises

20

(i) transforming the cells of said population with a gene coding for a cytotoxic polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4  
25 ParE polypeptide, the gene coding for the prophage P1 Doc polypeptide and a gene coding for a functionally equivalent polypeptide for anyone of said polypeptides, said gene is expressible in the cells of the population, and operably linked to the gene, a regulatory DNA sequence being regulatable by an environmental factor and which regulates the expression of said gene, and

30

(ii) cultivating the cell population under environmental conditions where the gene coding for the cytotoxic polypeptide is expressed, the expression leading to an at least partial killing of the cell population.

81. A method according to claim 80 wherein the survival of the cell population is limited in a first environment in which the gene is expressed, said cell population thereby being contained in said first environment.

5 82. A method according to claim 80 wherein the survival of the cell population is not limited when present in a first environment, which first environment could change to a second environment physically and/or chemically distinct from the first environment, in which first environment the gene whose expression results in the formation of a cytotoxically active polypeptide is not expressed, but the survival of which cell population  
10 is limited when transferred to a second environment or when present in a physically and/or chemically changed first environment, where the gene is expressed.

83. A method according to claim 80 wherein the survival of a cell population is being limited by providing in the cells a gene coding for a cytotoxic polypeptide which is operably linked to a nucleotide sequence encoding an antitoxin repressor substance  
15 which can undergo a decay when said cells are released to the outer environment to an extent whereby the repressor substance is converted to a non-functional form, whereby as a result of said decay, the function of the cells of the population will be gradually limited.

20

84. A method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is naturally transferable to a second kind of cell, which method comprises providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a cytotoxic polypeptide selected from the  
25 group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides to an extent whereby the function of the cell is being limited, said first kind of cells having or being modified to have a chromosomal replicon comprising a regulatory  
30 nucleotide sequence the gene product of which inhibits the expression of said gene or the cell function-limiting effect of the polypeptide and thereby protects said first kind of cells, said regulatory gene being lacking in said second kind of cell, whereby, if a cell of the second kind receives said extrachromosomal recombinant replicon said gene is expressed and has a function-limiting effect on said second kind of cell.

85. A method according to claim 84 wherein the gene product which inhibits the expression of the expression of the gene coding for the polypeptide or the cell function-limiting effect of the polypeptide is selected from the *E. coli* relB polypeptide, the plasmid F CcdA polypeptide, the plasmid R1 PemI polypeptide, the plasmid RP4 ParD polypeptide, the prophage P1 Phd polypeptide and a functionally equivalent polypeptide of anyone of such polypeptides.

86. A method of stochastically limiting in an environment the survival of a cell population, the method comprising transforming the cells thereof with a recombinant replicon containing a regulatably expressible gene which, when expressed in a cell encodes a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides, the expression of said gene leading to formation of the polypeptide to an extent whereby the function of the cells is being limited, the expression of said gene is stochastically induced as a result of recombinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cells, inhibits expression of the gene coding for the polypeptide, said negatively functioning regulatory nucleotide sequence being contained in the recombinant replicon or in an other recombinant replicon present in cells of the population containing the replicon.

87. A cell according to claim 51 that is an animal cell.

88. A cell according to claim 87 that is selected from the group consisting of a mammal cell, a human cell and an insect cell.

89. A method according to any one of claims 1, 26, 32, 80, 84 or 86 wherein the first kind of polypeptide inhibits translation.

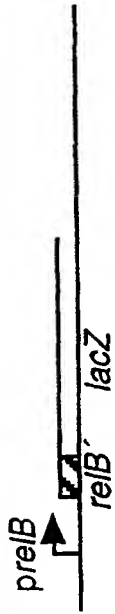
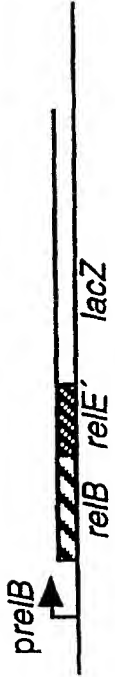
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pKG4002		28	45

Fig. 1



2/22

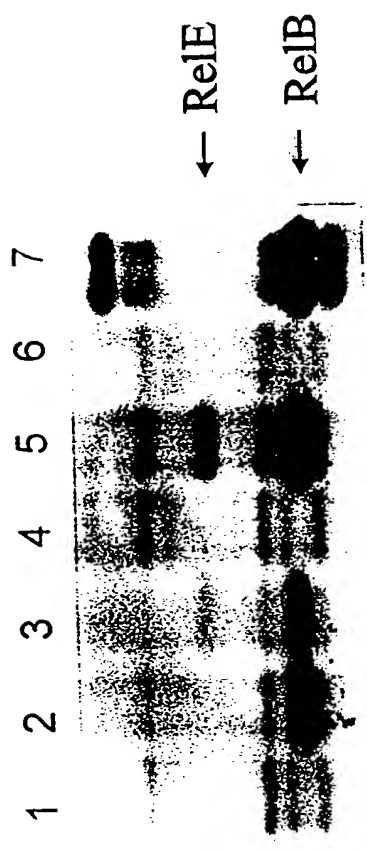


Fig. 2

3/22

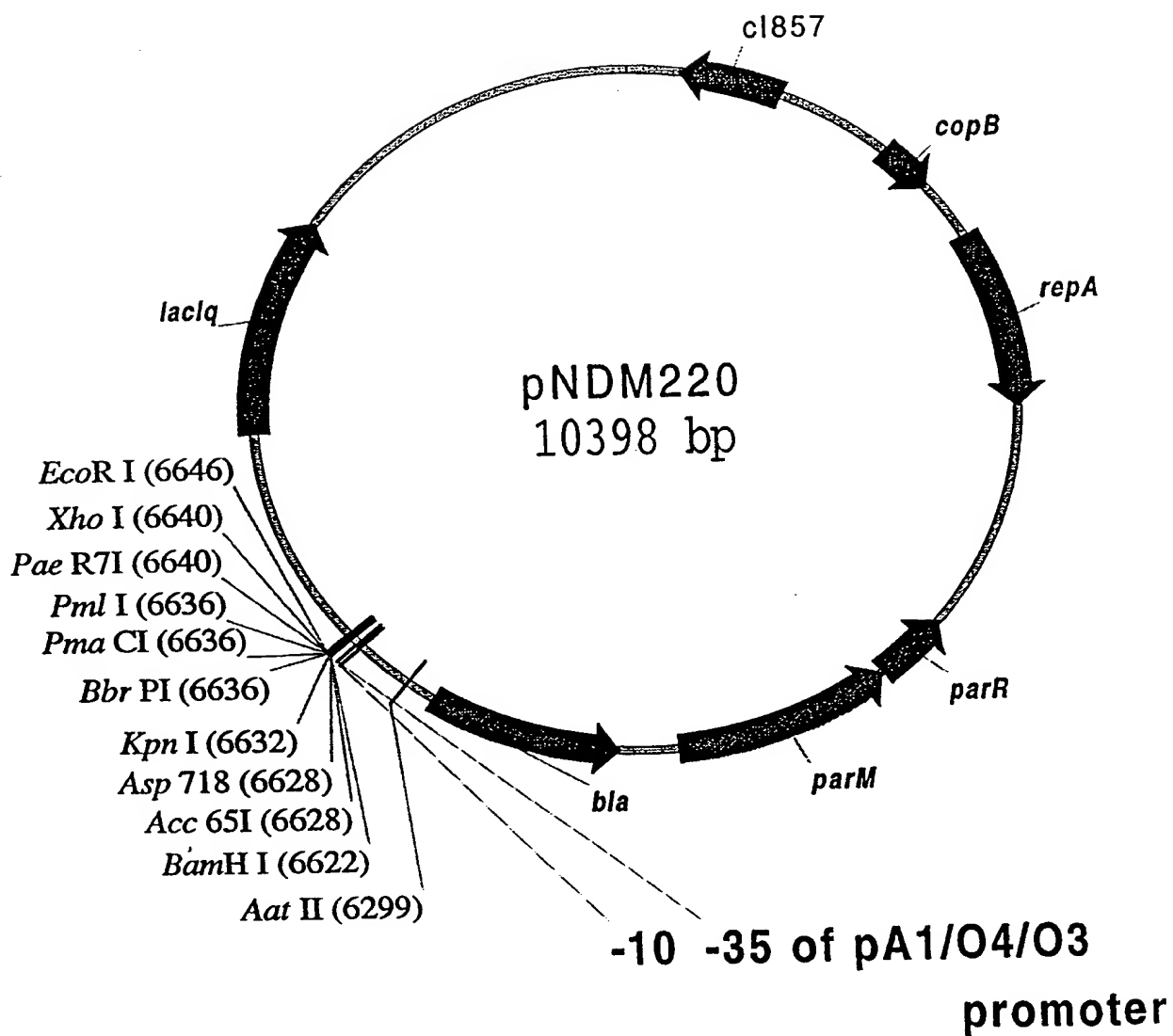


Fig. 3

4/22

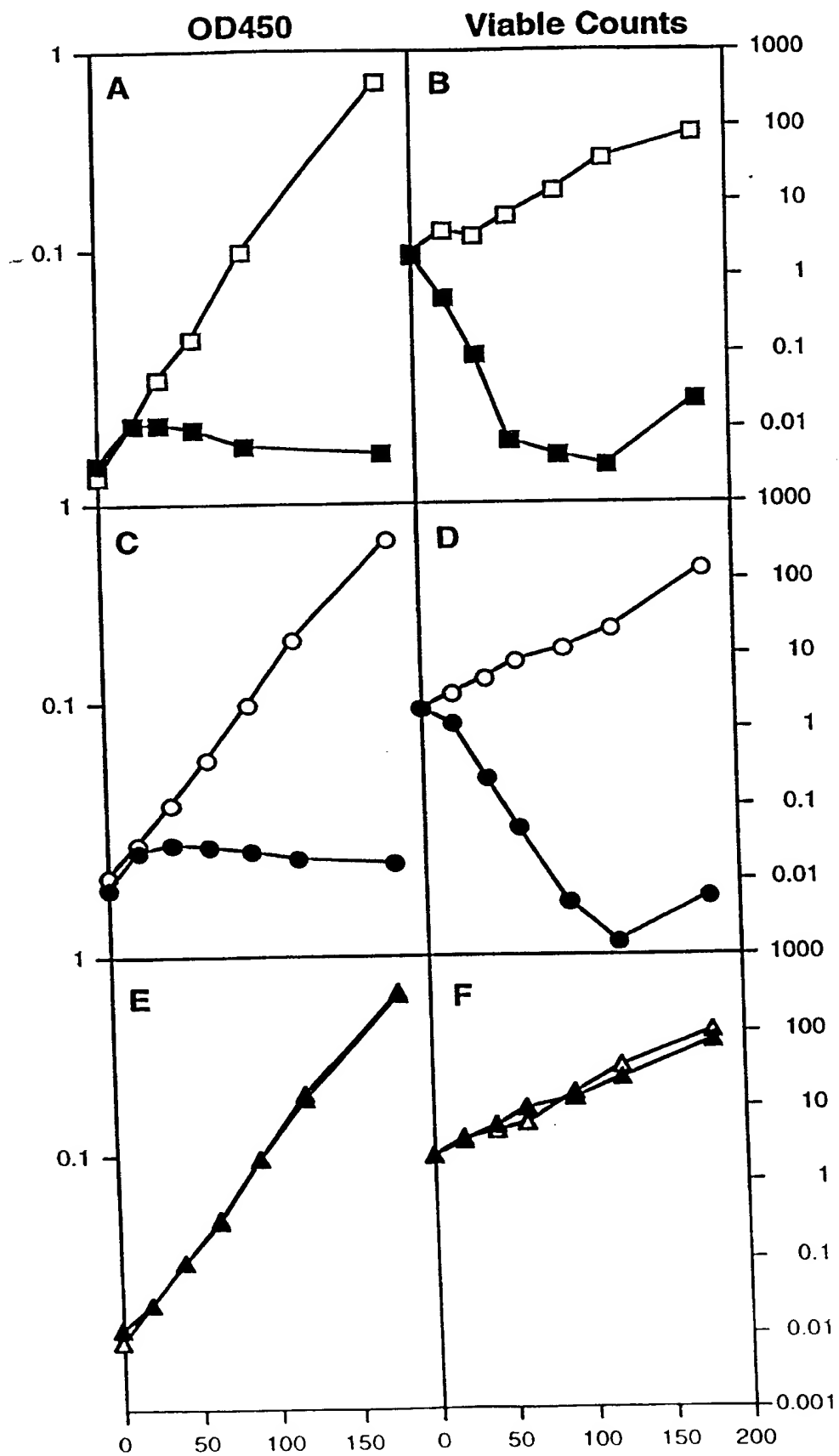


Fig. 4

5/22

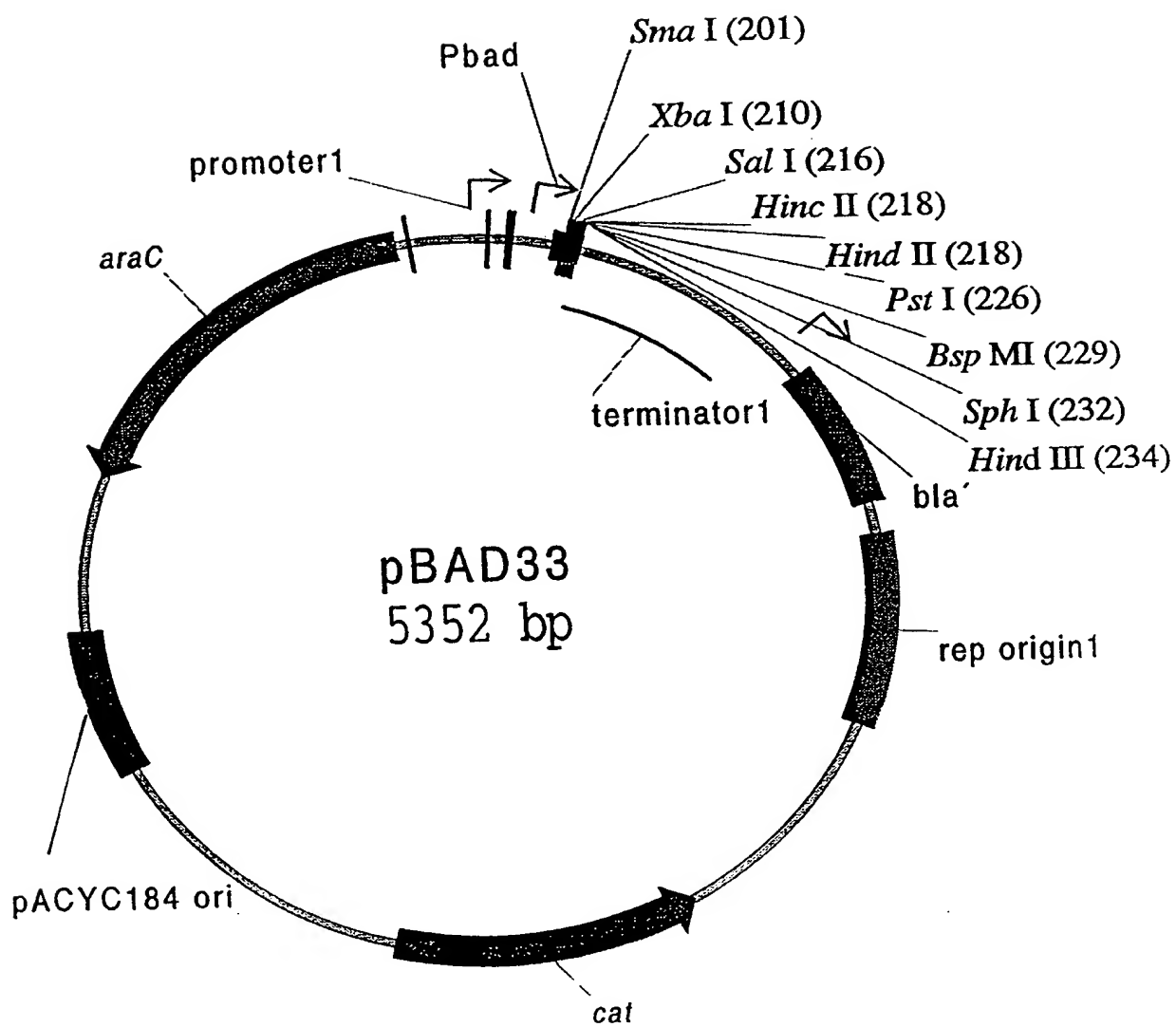


Fig. 5

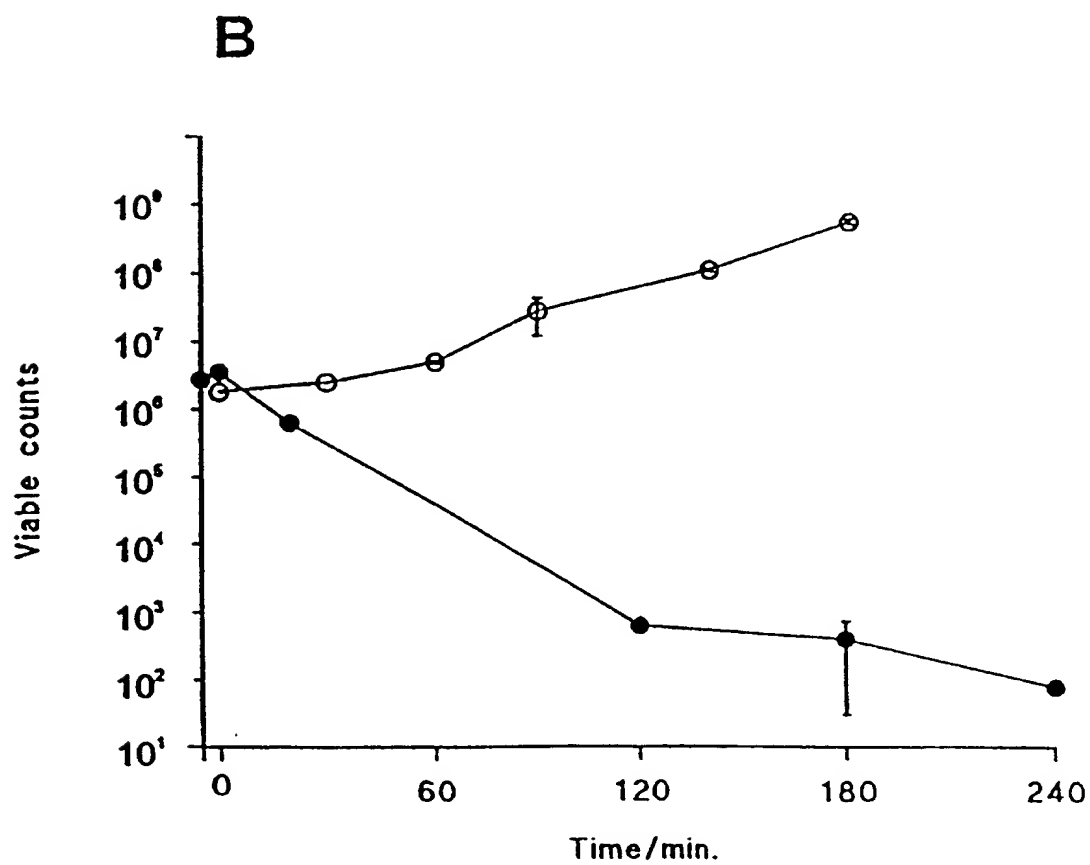
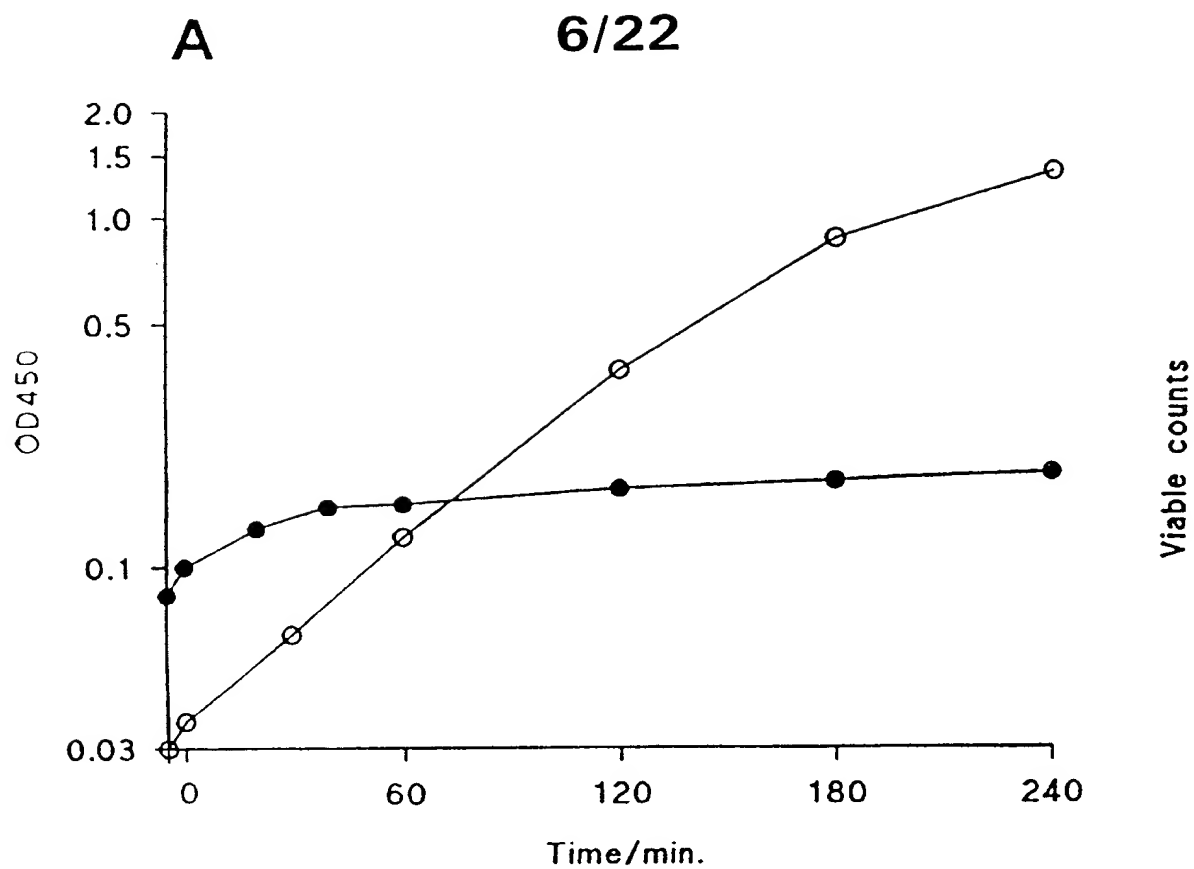


Fig. 6A

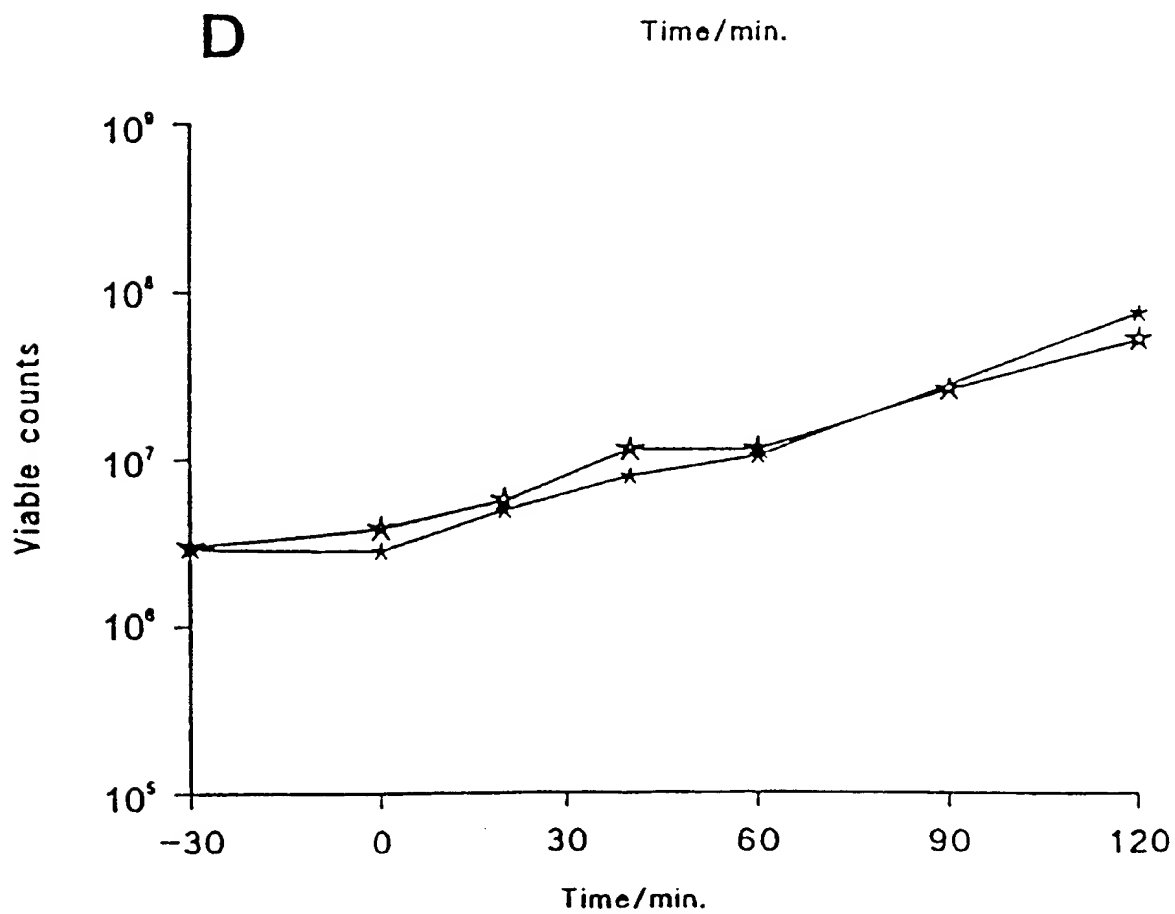
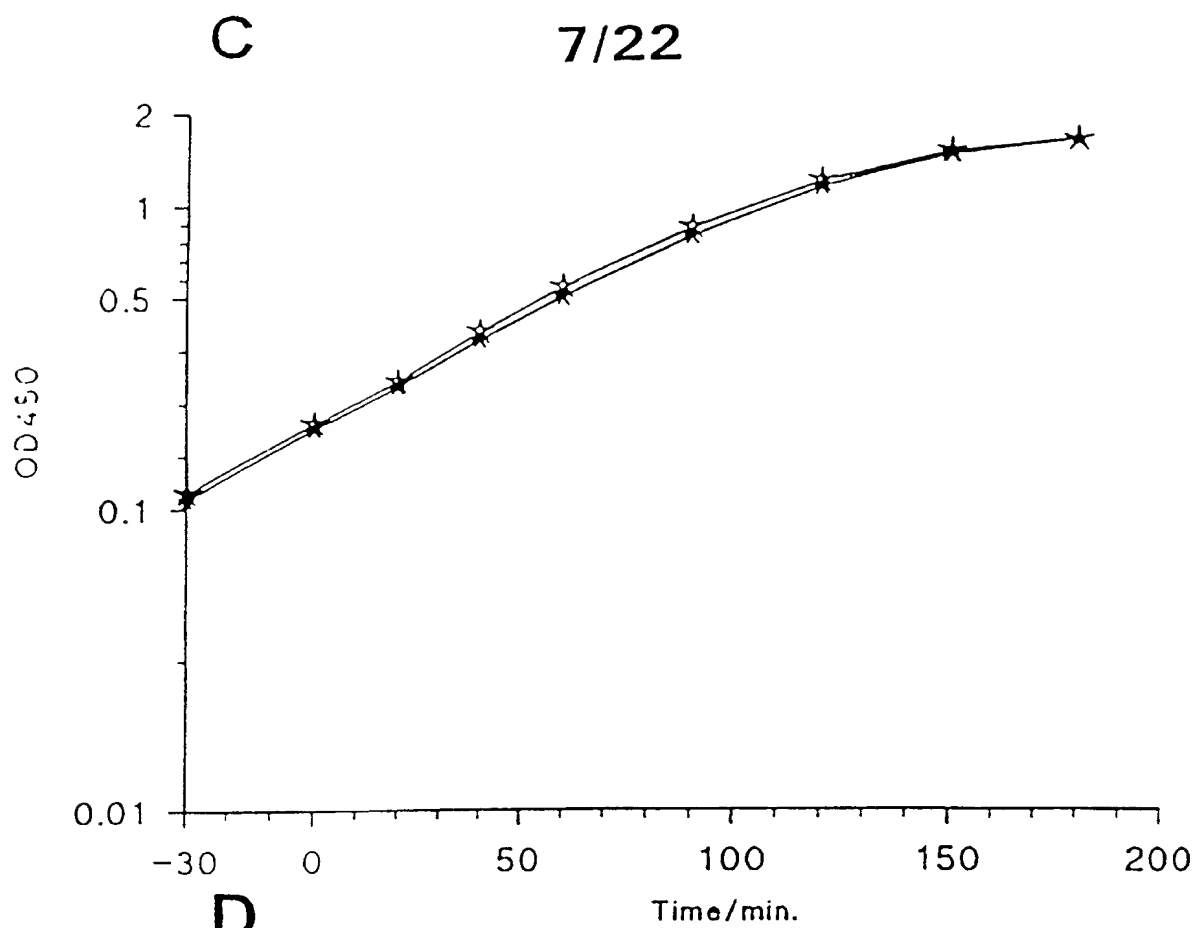


Fig. 6B

8/22

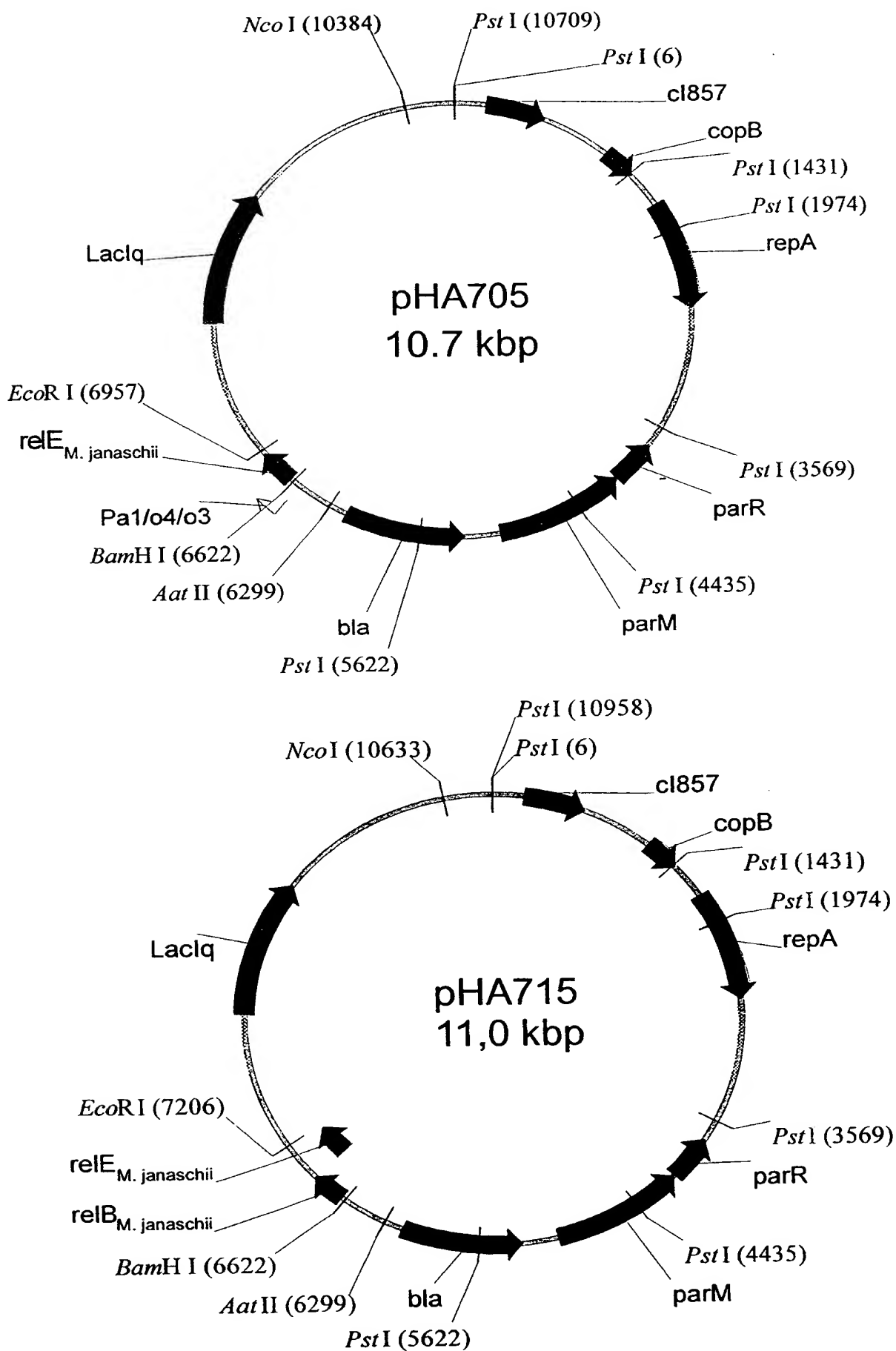


Fig. 7

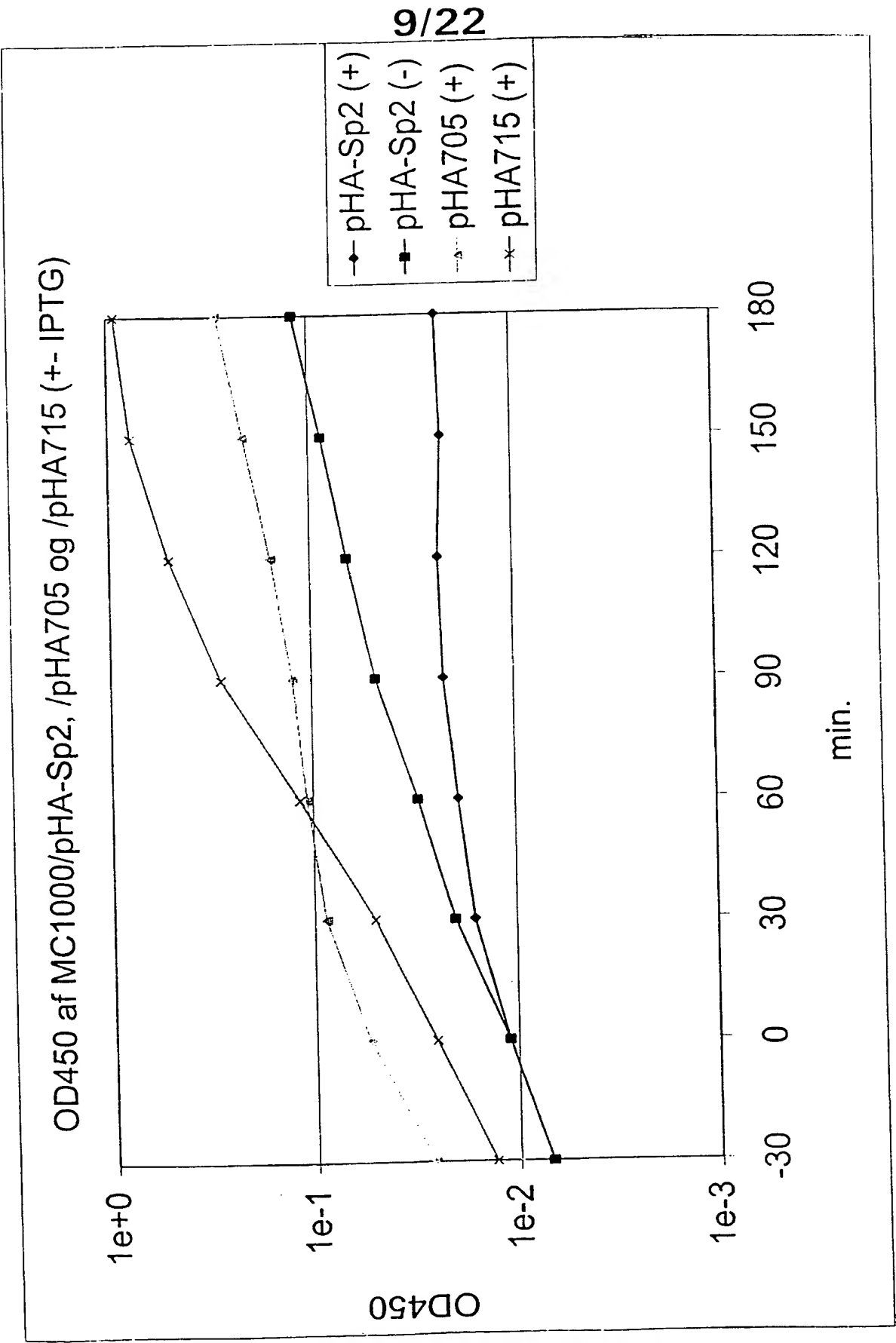


Fig. 8



10/22

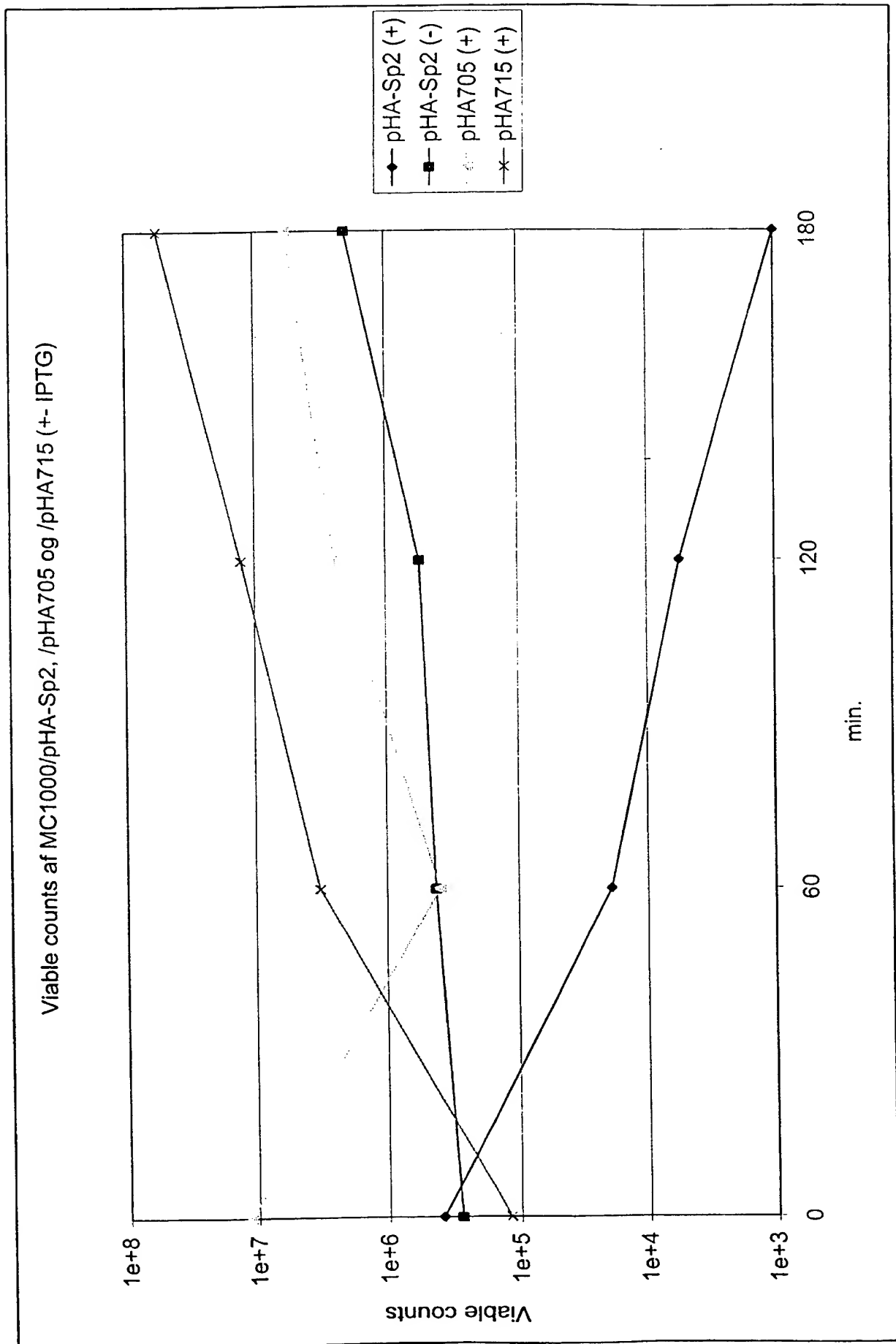


Fig. 9

11/22

DNA sequence of the *relB<sub>sp2</sub>* locus of *S. pneumoniae* (reverse complement of part of contig 80 in the tigr database)

```

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151 ATGGGTAAAA CCAAACCTT TGGAGATATC GACATCGAGA GTTTTGGTGT
201 AAGCCATGAT GCAGTCGCAC CGCAGTTCTA TCGCTTTATG AAGGATGATA
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651 TGTTCTTTTT ATATTGACTG AACACCTAAA AAGTAATACA ATGGTGTTAC

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751 GAAGCTGATA AAACATTTAT GAAAGCAATG GCTAAGTTTG AAGGAGTTTC
801 CCTTTCGGAA CTTATTCGCA CAAAACCTCT TGAAGCCCTA GAAGATGAAT
851 ACGATGCTCG TGTGGCAGAT TTAGCCTATC AAGAGTATTT AGAAGACTTG

      SD
901 GAAAAAGGAG TTGAACCCAT TACTTGGGAA GAAATGATGC ATGATTTTAGG

      Start relEsp2/End relBsp2
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      End relEsp2
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Protein sequences:

*RelB<sub>sp2</sub>* (pHi=4.2):

mttitlkvse adktfmkama kfegvslsel irtktleale deydarvadl ayqeyledle  
kgvepitwee mmhdlglkde

*RelE<sub>sp2</sub>* (pHi=11.1):

mnnyklvpt rrfikqlkkl drytqklitn ylqtnvledp rrrhgkalvgn  
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Fig. 10

12/22

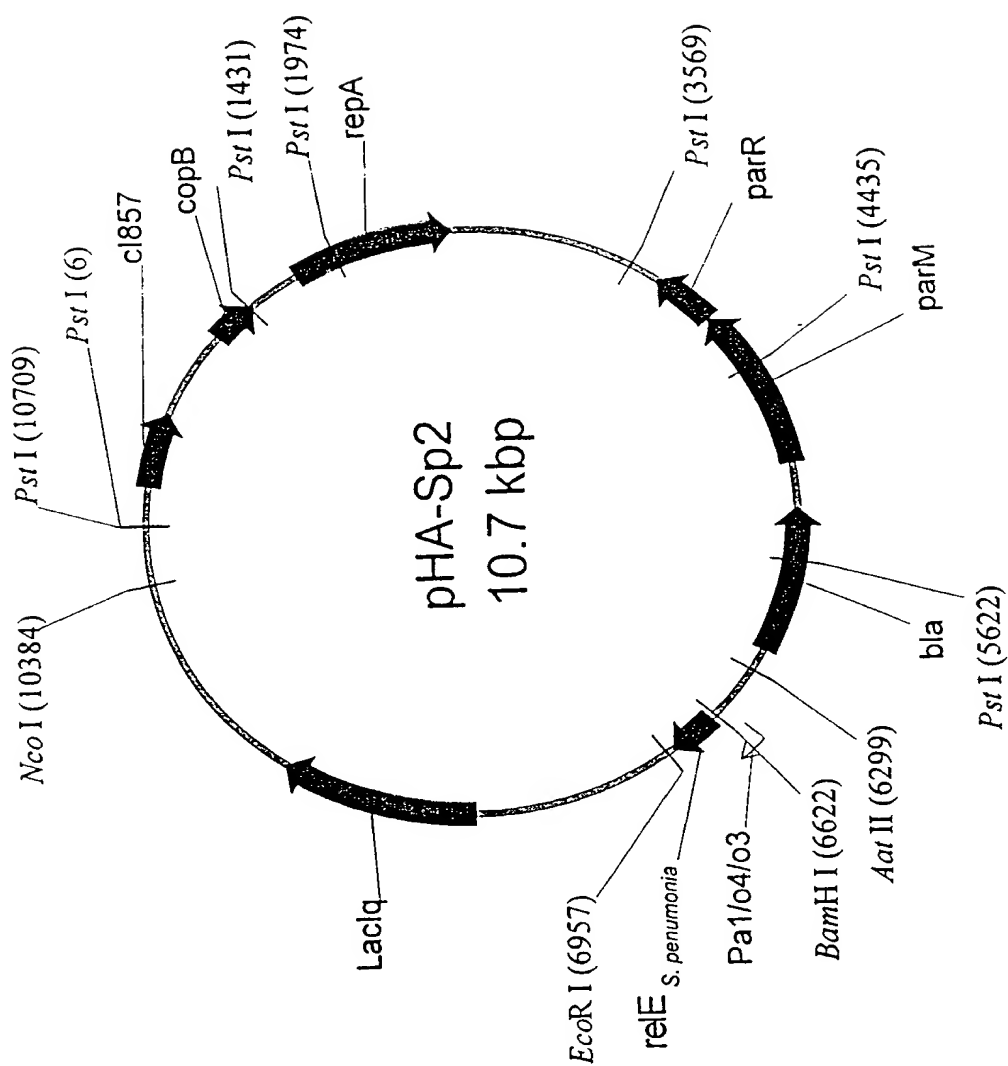


Fig. 11

13/22

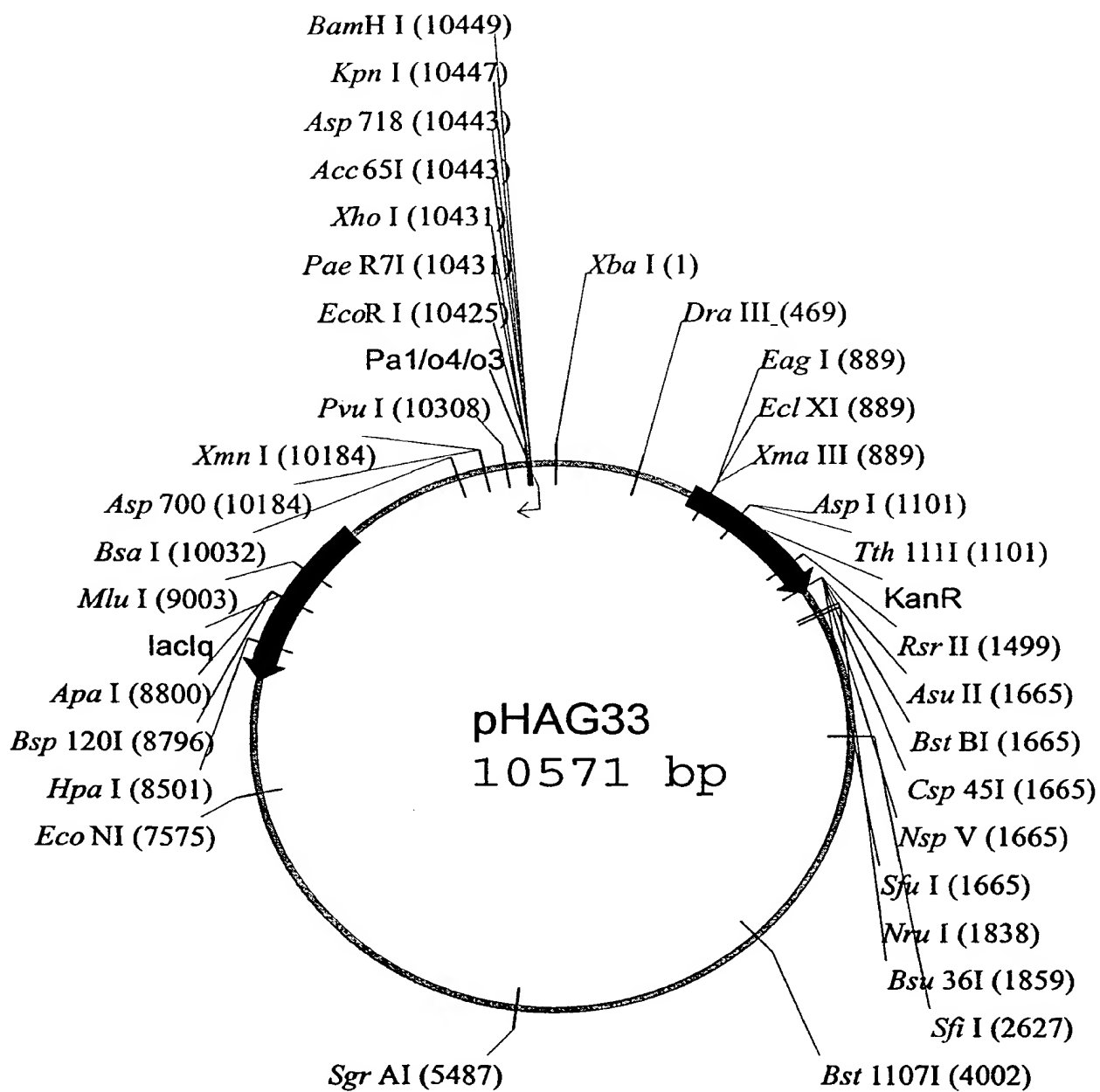


Fig. 12

14/22

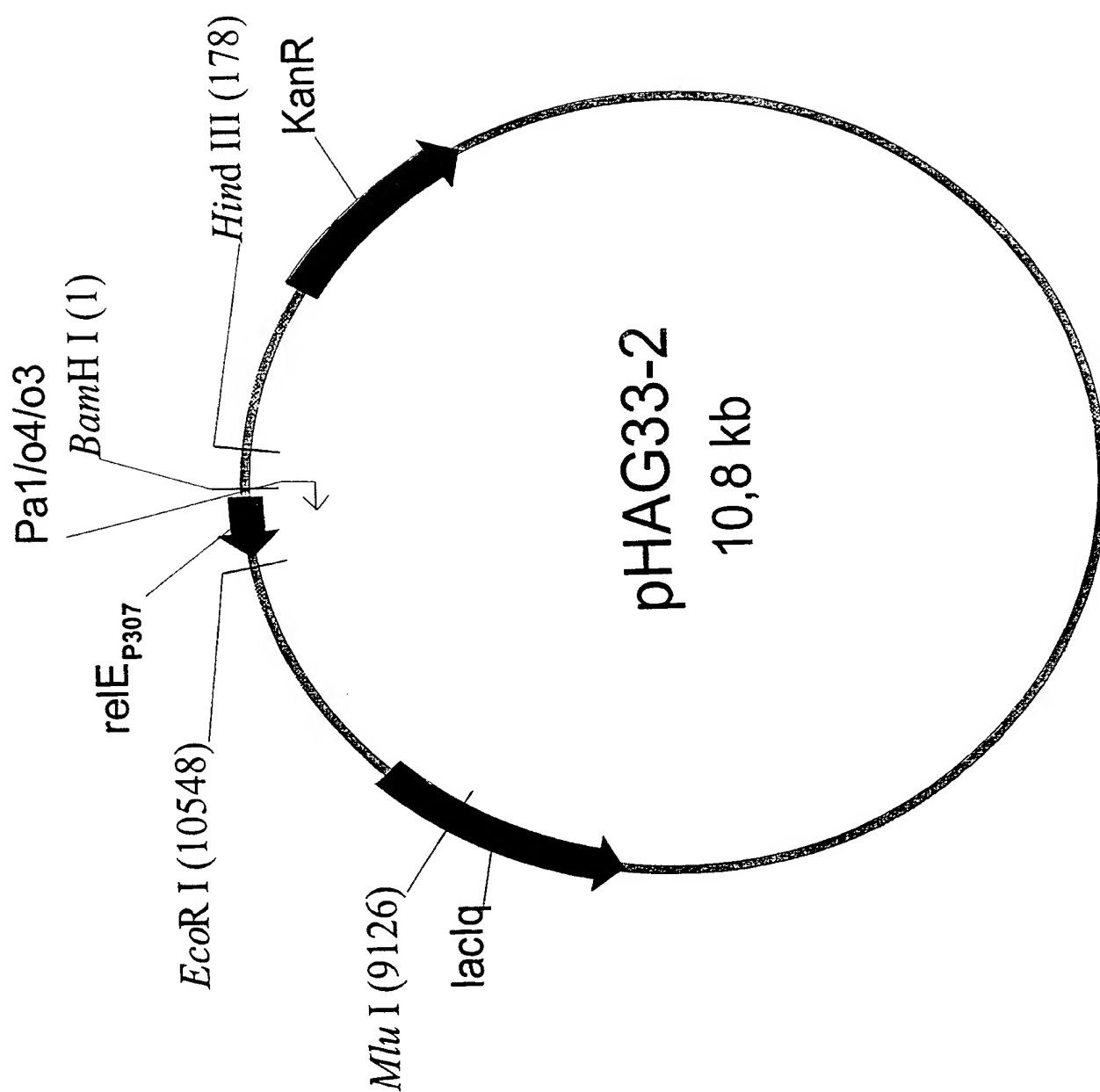


Fig. 13

15/22

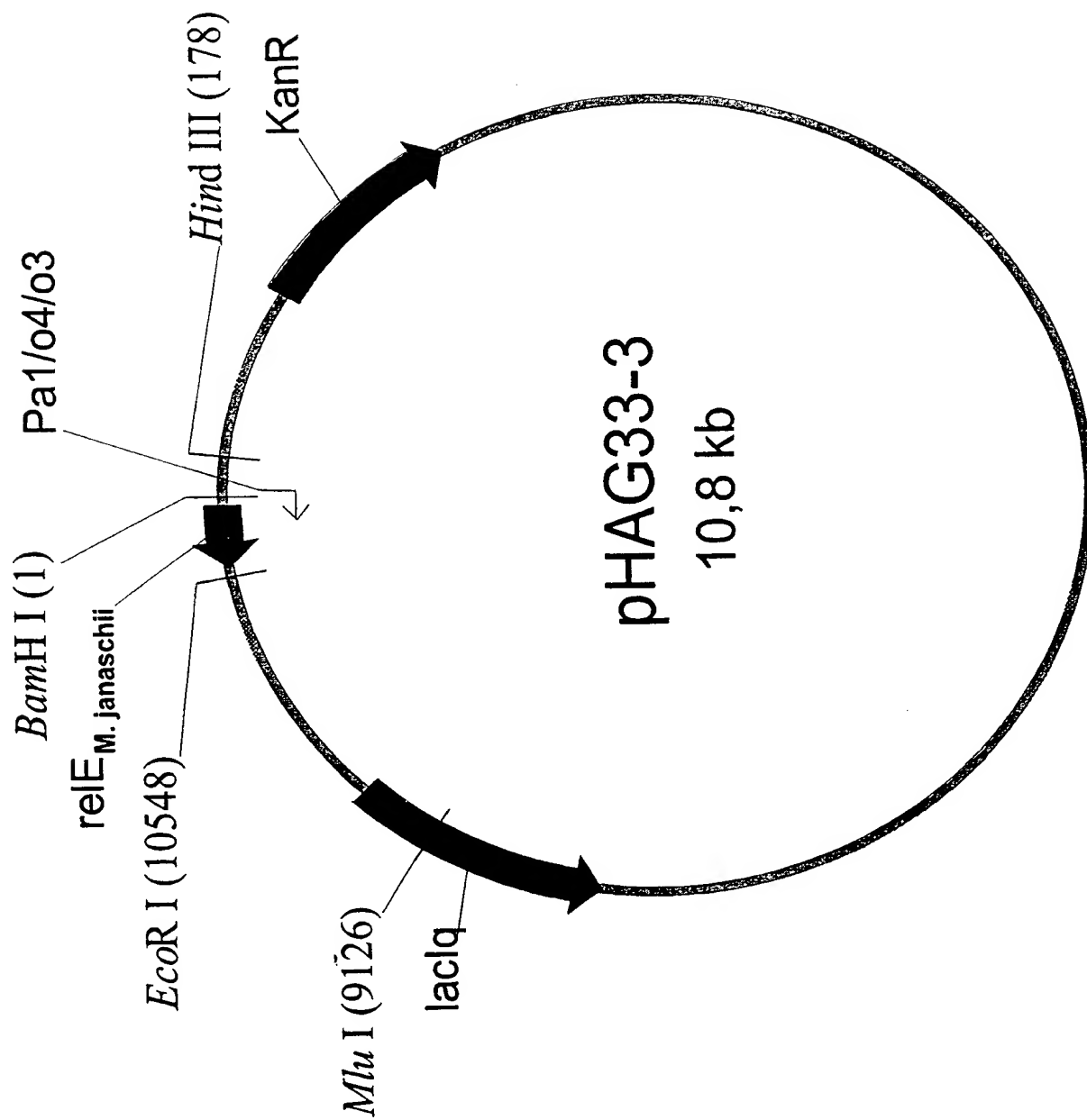


Fig. 14

16/22

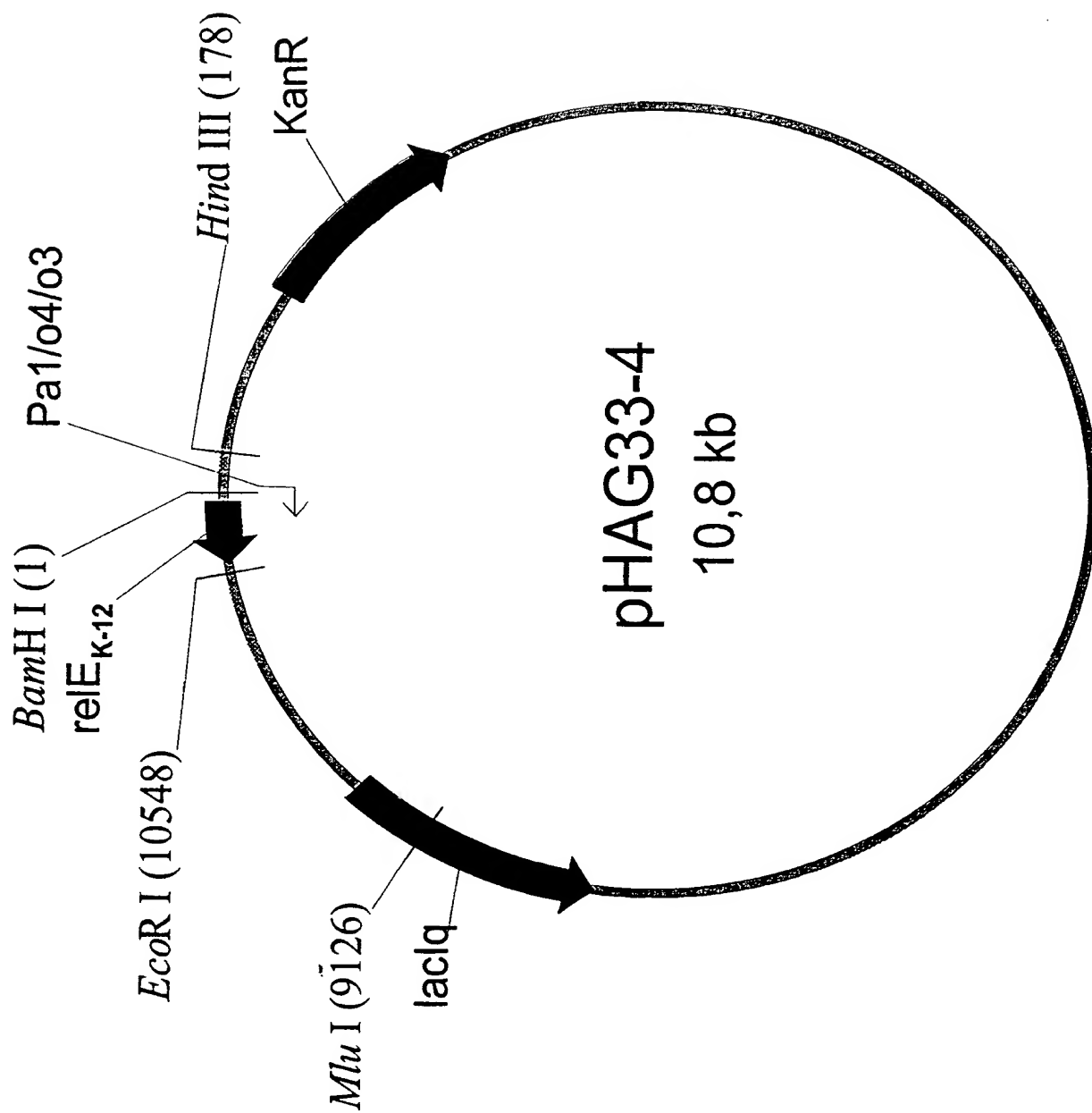


Fig. 15

17/22

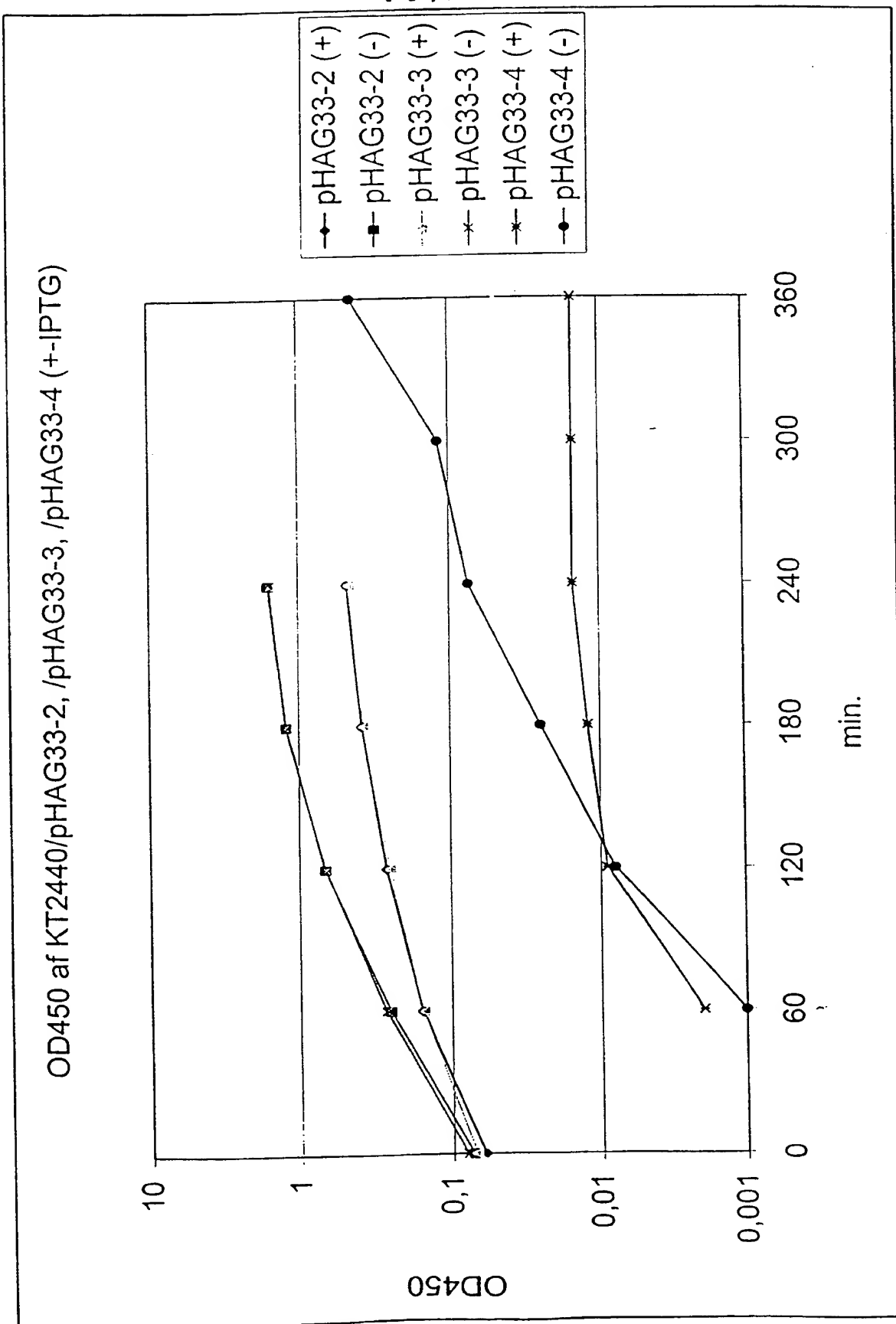


Fig. 16



18/22

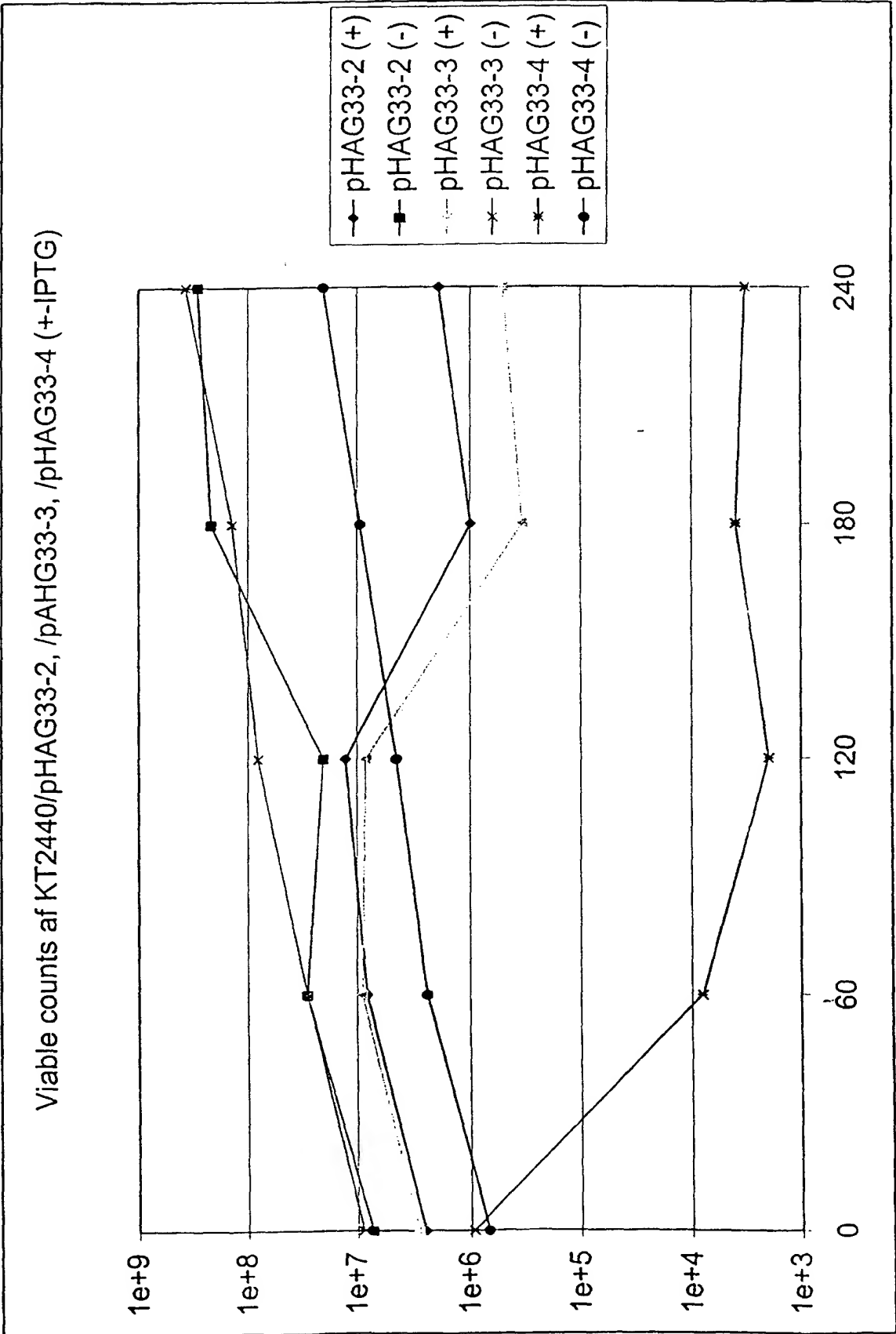


Fig. 17

19/22

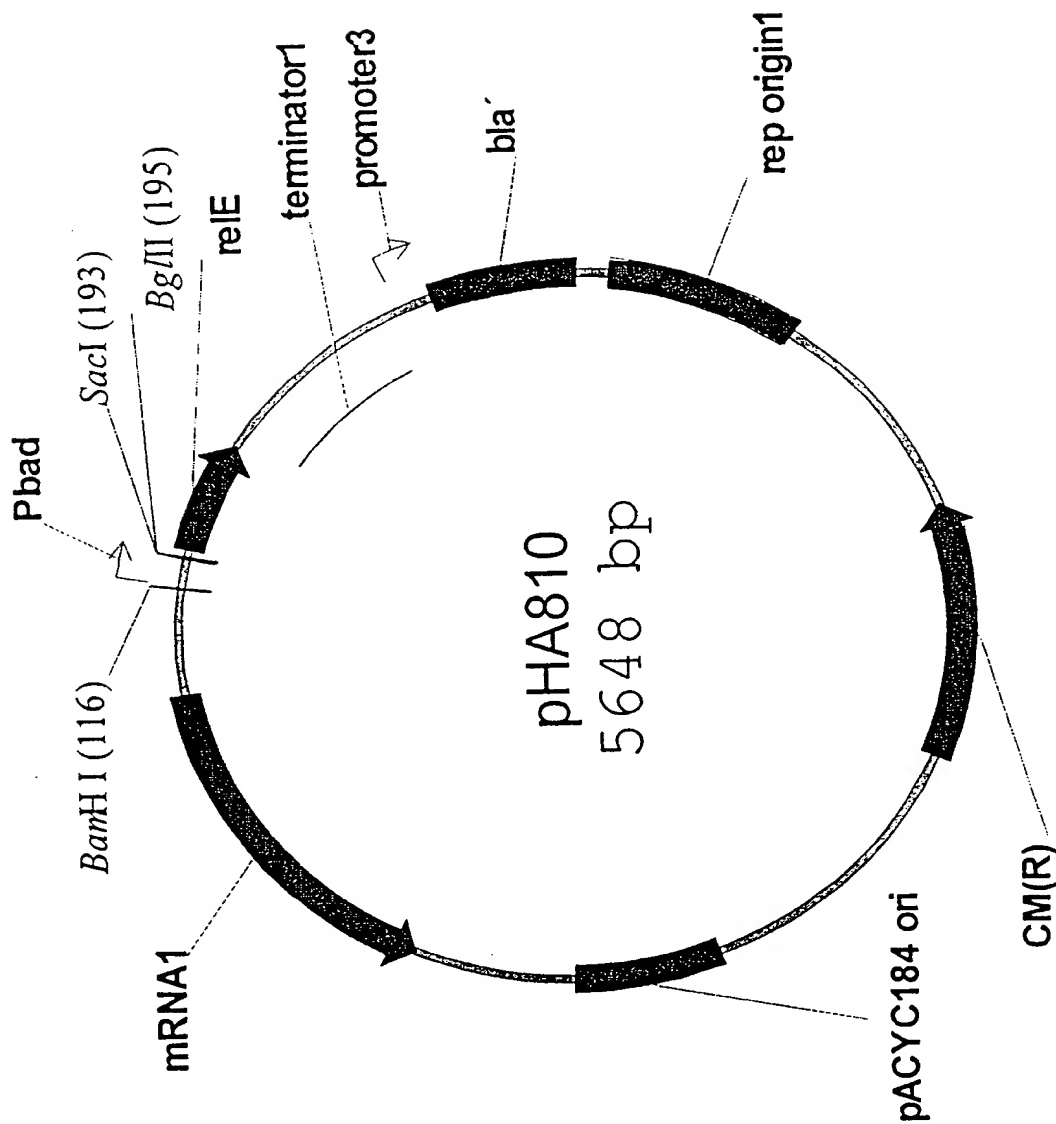


Fig. 18

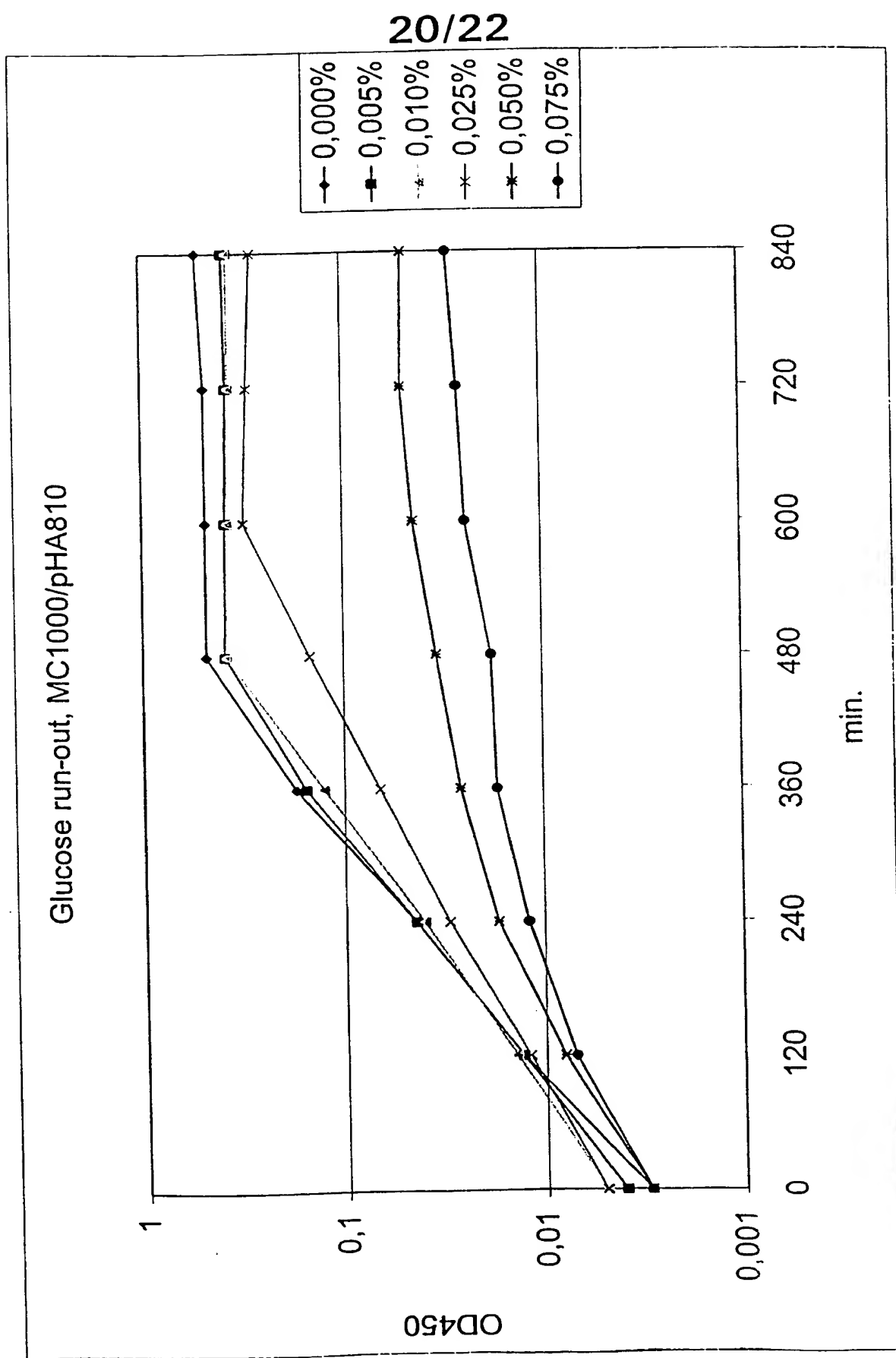


Fig. 19

21/22

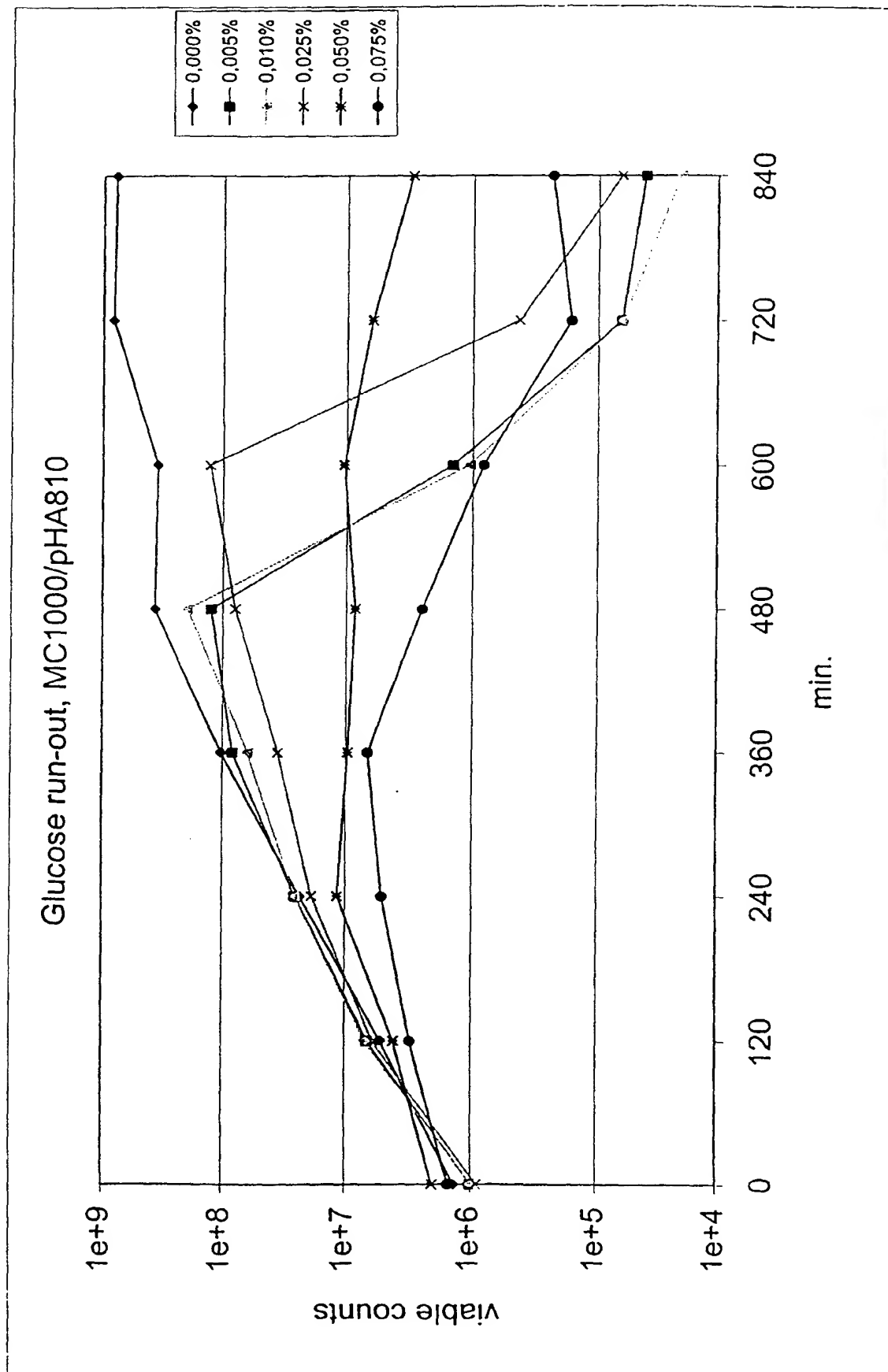


Fig. 20

22/22

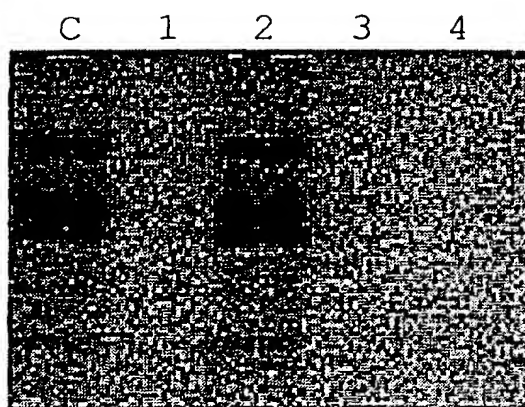


Fig. 21

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            <220>  
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                    a PCR-fragment from pNZ945

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5 <210> 4  
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10 <220>  
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20 <210> 5  
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25 <220>  
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gene relEP307 from pNZ945

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35 <210> 6  
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40 <220>  
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45 <400> 6  
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50 <210> 7  
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65 <210> 8  
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70 <220>  
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a DNA fragment encoding relEP307 by PCR

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	tttctgccgc atgcgggtgt tgcataaaac gtgttacgtt cctttatcga caggtcaggt	180	
	caccgctcac ccgcccacga gaaagcaaca ctgacatgct aaagcaaaaa atagatgaat	240	
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	gatgatggcc ggactcttta aaaacgagct gacctgcaca atacaggatg gacttagcaa	360	
	tggctgctcc tggcacaaag cggacagtga tcaccgttct tacgactact ttctgacttc	420	
	cttcgtgact tgccctaagc atgtttagt rbmnrarbst artgcgatac ttgtaatgac	480	
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	agtgatgaag atgctgaact tgtggagata gtgaaagaac ggcttcgtaa tcctndrbst	720	
	artraagcca gtacgtgtga cgtcggatga actctgatgg cgtattttct ggattttgac	780	
	gagcgggcac taaaggaatg gcgaaagctg ggctcgacgg tacgtgaaca gttgaaaaag	840	
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	gaggndrcgg tcaaacgcat tctctgaacc aaagcatgac atctctgttt cgcaccgsta	1080	
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	cgaggaaaga cctctgcgag gtacgaatcc gaaccgndhk caccagacgg aggtcgctgt	1260	
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	35 40 45		
	Gly Tyr His Asn Tyr Tyr Arg Ile Arg Val Gly Asn Tyr Arg Ile Gly		
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	Lys Ser Ile Tyr Asp Tyr Phe Pro		
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     Glu Glu Ile Pro Asn Lys Asn Asn Pro Pro Glu Ile Pro Asn Val Lys  
 15                      35                    40                    45  
     Lys Leu Lys Gly Ala Asp Ser Tyr Tyr Arg Ile Arg Val Gly Asp Tyr  
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     Arg Ile Gly Phe Lys Tyr Glu Asn Gly Lys Ile Val Phe Tyr Arg Val  
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     Lys Thr Asp Pro Ile Pro Trp Lys Arg Phe Asp Val Lys Lys Ile Glu  
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                     50                    55                    60  
 40 Tyr Phe Leu Asp Lys Pro Thr Lys Thr Val His Ile Leu Lys Val Glu  
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5

Lys Gly Ser Asp Glu Val Tyr Arg Val Arg Ile Gly Lys Phe Arg Val  
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 Ser Met Lys Gly Tyr Thr Glu Leu Tyr Arg Leu Arg Ile Gly Thr Phe  
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 35 40 45  
 Asp Cys Tyr Lys Ile Lys Leu Arg Ala Ser Gly Phe Arg Leu Val Tyr  
 50 55 60  
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 35 40 45  
 Asp Cys Tyr Lys Ile Lys Leu Arg Ser Ser Gly Tyr Arg Leu Val Tyr  
 50 55 60  
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 Arg Leu Glu Asn Pro His Val Pro Ser Ala Lys Leu Ser Gly Ala Glu  
 35 40 45  
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 Leu Ala Gly Thr Phe Ser Ala Arg Arg Gly Thr Tyr Arg Leu Leu Tyr  
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           20                  25                  30  
 15 Phe Val Phe Gly Pro Leu Leu Asn Asn Pro His Arg Leu Gly Lys Pro  
       35                  40                  45  
       Leu Arg Asn Asp Leu Glu Gly Leu His Ser Ala Arg Arg Gly Asp Tyr  
           50                  55                  60  
       Arg Val Val Tyr Ala Ile Asp Asp Gly His His Arg Val Glu Ile Ile  
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 40 Pro Glu Asn Tyr Gln Asp His Ala Leu Val Gly Glu Trp Lys Gly Tyr  
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           65                  70                  75                  80  
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 Met Thr Leu Leu Ile Asn Asn Thr Leu Pro Leu Pro Ala Val Tyr Lys  
 35 40 45  
 Asp His Pro Leu Gln Gly Ser Trp Lys Gly Tyr Arg Asp Ala His Val  
 50 55 60  
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 35 40 45  
 30 Leu Lys Gly Lys Trp Lys Pro Tyr Arg Glu Cys His Ile Lys Pro Asp  
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 Val Glu Glu Ile Ile Lys Gln Pro Glu His Tyr Lys Pro Leu Arg Gly  
 35 40 45  
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 Asp Val Gln Pro Ile Ile Glu Ala Leu Gln Lys Gly Val Ile Ser Gly  
 35 40 45

10

Asp Arg Leu Ala Gly Phe Gly Ser Asp Ile Tyr Val Tyr Lys Leu Arg  
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 Ile Lys Asn Ser Asn Ile Gln Lys Gly Lys Ser Ser Gly Tyr Arg Leu  
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 5 Ile Tyr Leu Leu Glu Ser Glu Asn Ser Ile Leu Leu Leu Thr Ile Tyr  
                                   85                                  90                                  95  
 Ser Lys Ala Glu Gln Glu Asp Ile Ala Ala Ser Asp Ile Asn Ser Ile  
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 25 Leu Val Arg Ile Thr Leu Thr Lys Val Ala Arg Glu Lys Ala Leu Pro  
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 Ala Val Leu Asn Arg Asn Glu Pro Ala Phe Tyr Cys Val Pro Ala Ser  
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 Thr Tyr Glu Ile Met Met Asp Lys Leu Glu Asp Leu Glu Leu Leu Ala  
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 Gly Ala Pro Ala Ala Val Leu Val Gly Ala Asp Glu Trp Glu Ser Leu  
 35 40 45  
 Gln Glu Thr Leu Tyr Trp Leu Ala Gln Pro Gly Ile Arg Glu Ser Ile  
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 Gly His Pro Ala Ala Val Leu Ile Ser Ala Asp Asp Leu Ala Ser Ile  
 35 40 45



## 12

Glu Glu Thr Leu Glu Val Leu Arg Thr Pro Gly Ala Ser Glu Ala Ile  
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14

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 35 Leu Ile Phe Ser Asp Ala Glu Phe Ile Asp Ser Ile Gln Arg Gly Leu  
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 35           50                  55                  60  
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           50                  55                  60  
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35 40 45  
20 Pro Asp His Glu Pro Leu Ser Thr Gln Glu Leu Glu Gln Leu Asn Ser  
50 55 60  
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 25           <210> 50  
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           <212> DNA  
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 30           <220>  
           <223> Primer relE-Sp2/cw was used for the  
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 40           <211> 34  
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 45           <223> Primer relE-Sp2/ccw was used for the  
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                   DNA of *S. pneumoniae*  
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           <211> 58  
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 55           <213> Artificial Sequence  
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           <223> Primer relEk12 was used for the PCR-amplification  
                   of DNA-fragments comprising genes relEK-12,

## releP307 and releMj

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                  <220>  
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                          and releMj

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20                   <210> 54  
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                  <213> Artificial Sequence

25                   <220>  
                  <223> Primer releP307 was used for the PCR-amplification  
                          of DNA-fragments comprising genes releK-12,  
                          releP307 and releMj

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                  <210> 55  
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35                  <213> Artificial sequence

                  <220>  
                  <223> Primer P4 was used for the PCR-amplification of  
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40                  and releMj

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45                   <210> 56  
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50                   <220>  
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20

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                    and relEMj

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15           <210> 58  
            <211> 24  
            <212> DNA  
            <213> Artificial Sequence

20           <220>  
            <223> Primer S-RelE was used for the PCR-amplification  
                    of the RelE coding region from the plasmid pMG223

25           <400> 58  
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30           <210> 59  
            <211> 23  
            <212> DNA  
            <213> Artificial Sequence

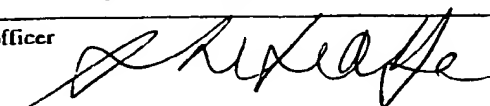
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            <223> Primer AS-RelE was used for the PCR-amplification  
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35           <400> 59  
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A. The indications made below relate to the microorganism referred to in the description</b> on page <u>33</u> , line <u>13</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b>	
Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH	
Address of depositary institution (including postal code and country) Mascheroder Weg 1B D-38124 Braunschweig Germany	
Date of deposit 30 April 1998	Accession Number DSM 12157
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<p style="text-align: center;">For receiving Office use only</p> <p><input type="checkbox"/> This sheet was received with the international application</p> <p>Authorized officer</p>	<p style="text-align: center;">For International Bureau use only</p> <p><input checked="" type="checkbox"/> This sheet was received by the International Bureau on:</p> <p style="text-align: center;"><b>13 SEP 1999</b></p> <p>Authorized officer </p>
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INDICATIONS RELATING TO DEPOSITED MICROORGANISMS  
(PCT Rule 12bis)

5

Additional sheet

In addition to the microorganism indicated on page 33 of the description, the following microorganisms have been deposited with

10 DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH  
Mascheroder Weg 1b, D-38124 Braunschweig, Germany

on the dates and under the accession numbers as stated below:

15

Accession number	Date of deposit	Description Page No.	Description Line No.
20 DSM 12158	30 April 1998	33	32
DSM 12159	30 April 1998	35	10
DSM 12160	30 April 1998	34	31
DSM 12161	30 April 1998	33	21

25

For all of the above-identified deposited microorganisms, the following additional indications apply:

30

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed

35 to be withdrawn.